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16. ABSTRACT

Crude oil was incubated with raw and sterile river water samples and assayed for mutagenic activity by the Ames test to determine the ability of naturally-occurring freshwater microorganisms to form mutagenic biodegradation products from crude oil. River water samples supplemented with inorganic salts were incubated with one percent crude oil in Erlenmeyer flasks for 1-6 weeks and 4 and 20 C with shaking. Replicate flasks were extracted at various time intervals with 1,1,2-trichloro-1,2,2-trifluoroethane, the solvent evaporated and the residues taken up in dimethyl sulfoxide for mutagenicity testing. The extracts were assayed for mutagenic activity by the plate incorporation method of the Ames test with Salmonella tester strains TA 100, TA 1535, TA 1537 TA 1538 and TA 98. Mammalian liver microsomes were not used in the mutagenicity test system as the oi-degrading microorganisms constituted the enzyme activation system in this study. Preliminary studies in Colorado with lagoon water and Black Hollow, Colorado crude oil showed that frameshift mutagens were produced during microbial degradation of the oil. Studies in Alaska with Chena River water and Prudhoe Bay, Alaska crude oil showed that both frameshift and base-pair substitution mutagens were present in the oil after microbial degradation. Mutagenic activity was not detected in either of the crude oils tested prior to incubation.. The presence of mutagenic products may be related to the presence of mutagenic products in microbiologically-degraded crude oil.

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This study is also filed as a thesis by the same title in the Morgan Library of Colorado State University, Fort Collins, Colorado 80523.

ABSTRACT

Crude oil was incubated with raw and sterile river water samples and assayed for mutagenic activity by the Ames test to determine the ability of naturally-occurring freshwater micro-organisms to form mutagenic biodegradation products from crude oil. River water samples supplemented with inorganic salts were incubated with one percent crude oil in Erlenmeyer flasks for 1-6 weeks at 4 and 20 C with shaking. Replicate flasks were extracted at various time intervals with 1,1,2-trichloro-1,2,2-trifluoroethane, the solvent evaporated and the residues taken up in dimethyl sulfoxide for mutagenicity testing. The extracts were assayed for mutagenic activity by the plate incorporation method of the Ames test with Salmonella tester strains TA 100, TA 1535, TA 1537, TA 1538 and TA 98. Mammalian liver microsomes were not used in the mutagenicity test system, as the oil-degrading microorganisms constituted the enzyme activation system in this study.

Preliminary studies in Colorado with lagoon water and Black Hollow, Colorado crude oil showed that frameshift mutagens were produced during microbial degradation of the oil. Studies in Alaska with Chena River water and Prudhoe Bay, Alaska crude oil showed that both frameshift and base-pair substitution mutagens were present in the oil after microbial degradation. Mutagenic activity was not detected in either of the crude oils tested prior to incubation.

The appearance of microbiologically-mediated mutagenic activity in crude oil was affected by incubation time and temperature, nutrient levels and seasonal variations in the river. Mutagenic activity reached maximum levels in the stationary phase of microbial growth on the crude oils. Detectable levels of mutagens could be produced by river water microorganisms in one week at 20 C and in 3-4 weeks at 4 C. Supplementation of river water samples with nitrogen and phosphorus salts was essential for the microbial production of mutagens from crude oil. Seasonal variations in the ability of freshwater microorganisms to produce mutagens from crude oil were related to changes in the amount of surface water runoff into the river.

Toxicity studies showed that the crude oils tested were lethal to the non-oil-degrading bacterium, Escherichia coli K-12, only during microbial degradation of the oil, indicating that toxic products were formed by the oil-degrading microorganisms. The presence of acutely toxic products may be related to the presence of mutagenic products in microbiologically-degraded crude oil.

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INTRODUCTION

Oil pollution in aquatic ecosystems has been recognized as a major environmental problem in recent years. Increased worldwide production, transportation and utilization of petroleum products has increased oil pollution so that few, if any, waters are unaffected. Major sources of aquatic oil pollution include oil refinery effluents, domestic and industrial wastes, oil tankers, offshore oil wells and natural oil seeps (258).

The Environmental Protection Agency (148) has stated that additional research is needed to determine the cumulative effects of oil on the aquatic food chain. The direct and indirect effects of oil pollution on man have not been fully evaluated. The acutely toxic, volatile components of oil have the greatest immediate impact on aquatic organisms. Although these components are rapidly removed by evaporation, they may cause extensive disruption of aquatic ecosystems (235). The non-volatile components of oil may be potentially carcinogenic (382, 384) or may be rendered more toxic by chemical and biological degradation (49, 206, 383). The concentration of toxic oil pollutants in water is usually too low to present an obvious health hazard to man or animals, except in highly polluted areas. Chronic exposure to low levels of toxic compounds may have significant long-term effects, however (176).

The majority of research on aquatic oil pollution has been conducted in marine and estuarine waters because of the pollution problems inherent in oil tanker and offshore drilling operations. Construction of overland transportation facilities for oil, such as the Alaska pipeline, has lead to increased research on the effects of oil pollution in soil and freshwater environments.

This project was designed to investigate the acute and genetic toxicity of crude oil to indicator organisms before and after degradation by naturally-occurring freshwater microorganisms. Acute toxicity was determined by monitoring the survival of a non-oil-degrading bacterium, *Escherichia coli* K-12, exposed to crude oil in the presence and absence of oil-degrading microorganisms. Crude oil was tested for changes in mutagenic activity during microbial degradation by the *Salmonella* mutagenicity test developed by Ames (13).

Preliminary studies were conducted at Colorado State University with Black Hollow, Colorado crude oil and lagoon water to develop a suitable test procedure. The project was then moved to the U. S. Environmental Protection Agency, Arctic Environmental Research Laboratory in College, Alaska. Water samples were obtained from the Chena River at various times of the year to determine the ability of naturally-occurring river water microorganisms to produce mutagenic biodegradation intermediates from Prudhoe Bay crude oil.

Incubation time and temperature, nutrient levels and seasonal variations in the river (water temperature, discharge measurements and heterotrophic bacterial numbers) were studied for their effects on the microbial production of mutagens from crude oil.

Prudhoe Bay crude oil was fractionated into saturate, aromatic and nitrogen-sulfur-oxygen (NSO) components by column chromatography and each fraction was tested for mutagenic activity before and after microbial degradation to determine which fractions were most mutagenically active.

The objectives of this study were as follows:

1. To test the ability of naturally-occurring freshwater microorganisms to mediate the production of mutagens in crude oil.
2. To establish the conditions most favorable for the production of mutagens in crude oil by microorganisms.
3. To determine the type of mutagens produced in crude oil during microbial degradation, i.e., frameshift or base-pair substitution mutagens.
4. To determine which fractions of crude oil contain the most mutagenic activity before and after microbial degradation.
5. To study the toxic effects of crude oil on a non-oil-degrading bacterium during microbial degradation of the oil.

6. To determine the usefulness of the Ames test for studying complex biochemical interactions which may increase the mutagenic burden in the environment.

LITERATURE REVIEW

The following review deals primarily with the interactions of petroleum in aquatic environments. The topics covered in this section include the extent and control of oil pollution, physical, chemical and biological alteration of oil, microbiological degradation intermediates of oil, toxic effects of oil, mutagenicity screening tests and media selection. The microbiological degradation of oil is emphasized to provide a literature base upon which the results of this investigation can be evaluated. Since the mechanisms of mutagen production by microorganisms growing on hydrocarbon substrates are poorly defined at present, an understanding of the events leading to mutagen production is essential for the proper interpretation of the results. The activation of some hydrocarbons to mutagens and/or carcinogens in mammalian systems is well known and some of this literature is included for comparative purposes.

Extent of Oil Pollution in Aquatic Environments

A dramatic increase in aquatic oil pollution has occurred within the last century. Prior to the development of petroleum as a major fuel source, the main hydrocarbon inputs into the environment were from natural oil seeps, pyrolysis products and primary biosynthesis by plants and animals (225). Hydrocarbon inputs from these sources were fairly constant from year to year and were easily controlled by natural degradation. Since the drilling of the first commercially successful oil well by Drake in 1859, the pollution of aquatic environments by oil has risen in proportion to its production (53).

Wakeham and Carpenter (347) studied the aliphatic hydrocarbon content of sediments from Lake Washington in Seattle and found that at depths below 30 cm the hydrocarbon content of the sediments was nearly constant at 30 μg/g. Above this depth, the concentration of aliphatic hydrocarbons steadily increased up to 1500 μg/g at the sediment surface. The depth at which the aliphatic hydrocarbon content of the sediments began to increase coincided with the time period in which the population of the area began to grow, about 1880. Stormwater runoff of lubricating oils and pyrolysis products from automobiles is a major source of hydrocarbon pollution in the lake.

The use of petroleum as a fuel was quite low before World War I. Nearly 96 per cent of the world's merchant marine fleet burned coal at that time, but by 1948, 75 per cent burned fuel oil. Only two ships of the U. S. naval fleet used oil, exclusively, in 1914, but by 1932, the entire fleet used fuel oil. Gasoline engines increased the demand for petroleum products during this same time period (53).

Various estimates of the amount of oil discharged into the oceans and rivers of the world have been made. Blumer (37) estimated that 5-10 million metric tons of oil were lost in the ocean in

1972. He was unable to account for two million metric tons of used lubricating oil. In a 1973 study, the National Academy of Sciences (258) estimated that over six million metric tons of oil entered the oceans of the world, annually.

Shipping operations are responsible for a great deal of the oil pollution in the oceans. Button (57) estimated that up to 0.1 per cent of the petroleum transported by water routes was spilled in the ocean. The total annual production of petroleum in 1974 was 3,800 million metric tons, of which 1,500 million were transported by water routes (153). Based on these figures, up to 1.5 million metric tons of petroleum was spilled in the ocean as a result of shipping operations in 1974.

Land-based discharges contribute more to aquatic oil pollution than shipping operations, according to the National Academy of Sciences (258). Approximately 530,000 metric tons of petroleum entered the ocean from U. S. rivers in 1973; the annual worldwide input of oil from rivers was 1.6 million metric tons. Farrington and Quinn (120) found that nearly 25 per cent of the organic material in wastewater effluents is hydrophobic in nature. They were able to distinguish between petroleum-derived hydrocarbons and animal fats and estimated that 28,000-140,000 metric tons of petroleum is discharged in wastewater effluents in the U. S. each year. Worldwide inputs of petroleum from wastewater have been estimated at 600,000 metric tons, annually.

Primary production of hydrocarbons by marine plants and animals is difficult to quantitate, but Farrington and Meyers (119) estimated that 1-10 million metric tons of hydrocarbons are produced naturally in the oceans each year.

The National Academy of Sciences (258) estimates of petroleum inputs into the ocean for 1973 and 1980 are shown in Table 1. An overall decline in oil pollution is expected by 1980, due to stricter regulation of discharges by ships and oil refineries. Development of offshore oil resources may increase oil pollution in the future. Land-based discharges and hydrocarbon inputs from natural sources are not expected to change much by 1980.

Control of Oil Pollution in Aquatic Environments

National and international legislation has been necessary to control oil pollution in the rivers and oceans of the world. Legislative attempts by the United States to control this widespread problem are reviewed in this section.

The Rivers and Harbors Act of 1899 (Refuse Act) was one of the first significant national efforts to control water pollution. Section 13 of this Act prohibited uncontrolled dumping of refuse into navigable waters and established a permit system to control waste disposal in water. The Refuse Act allowed for control of oily waste disposal, but

Table 1. Estimate of worldwide petroleum input into the oceans each year from various sources (258).

Source	Estimated oil input per year (metric tons) 1973	1980
Land-based discharges		
Refineries	200,000	20,000
Waste oils, runoff, sewage	2,500,000	2,400,000
Marine operations		
Tankers, using LOT*	310,000	200,000
Tankers, not using LOT	770,000	-
Drydocking	250,000	300,000
Bilge discharges	500,000	-
Marine accidents	300,000	150,000
Offshore production	80,000	200,000
Natural oil seeps	600,000	600,000
Atmospheric fallout	600,000	600,000
Total	6,110,000	4,470,000

*LOT: Load-on-top

lacked specificity with regard to oil pollution and protected only a limited number of waterways (147).

The Oil Pollution Act of 1924 was designed to deal specifically with oil pollution in navigable waters of the U. S. and closely followed the Oil in Navigable Waters Act enacted by the British in 1922 (382). This Act regulated the discharge of oil by tankers in coastal areas, but was limited to the control of tankers within a specified size range and did not have authority over large tankers.

As a result of the International Convention for the Prevention of the Pollution of the Sea by Oil held in London in 1954, the U.. S. ratified the Oil Pollution Act of 1961 (147). This Act was complementary to the Oil Pollution Act of 1924 and extended national power to regulate large tankers. Petroleum discharges were prohibited within coastal waters and the amount of oil discharged at one time on the high seas was limited. Inland water pollution control legislation was not sufficient, however.

The Federal Water Pollution Control Act of 1972 and its subsequent amendments have filled many of the gaps in water pollution control legislation. Section 311 of this Act deals specifically with oil pollution and prohibits the discharge of petroleum into navigable waters of the U. S. The U. S. Environmental Protection Agency was established by this Act to administer and enforce its provisions. The permit system established by the Refuse Act in 1899 to regulate waste disposal in water was replaced by the National Pollutant Discharge Elimination System (NPDES) which is designed to eliminate all pollutant discharges into navigable waters by 1985 (147).

The Federal Water Pollution Control Act of 1972 did not adequately protect all water sources, though, and the Safe Drinking Water Act of 1974 was enacted to control pollution of potable waters. Prior to this Act, authority to monitor drinking water for hazardous substances was limited to interstate shipment of water and applied only to the spread of communicable diseases, as required by the Public Health Service Act of 1962. The Safe Drinking Water Act of 1974 provides authority to monitor drinking water supplies for toxic chemicals and potentially carcinogenic substances, as well as communicable diseases, and is not limited to interstate regulation. The Act also gives the EPA authority to establish safe levels of chemicals in drinking water (338).

In addition to federal legislation, many states have enacted legislation dealing with local oil pollution problems, especially those states bordered by coastal waters.

Alteration of Oil in Aquatic Environments

Oil in aquatic environments undergoes compositional changes as a result of physical, chemical and biological processes. The effects of these alterations on oil are discussed below.

Physical Alterations

Physical alteration (weathering) of oil in water results in the loss of certain components from the oil by evaporation or dissolution, but does not change the molecular structure of its components. Compositional changes in oil by physical processes are governed primarily by the boiling points and solubilities of the components originally present in the oil. Many of the early changes in the composition of oil in water are due to weathering.

Smith and MacIntyre (307) studied the weathering of fuel oils in water and found that the rate of evaporative loss was proportional to the initial percentage of volatile components with boiling points lower than 270 C. Gordon, et al. (145) showed that evaporation accounted for the greatest weight loss of crude oil spilled on water in outdoor tanks. The evaporative loss of nonane, a C_9 paraffin, and cumene, a C_9 aromatic, from oil slicks in the ocean was compared by Harrison, et al. (155). Although the vapor pressures of these compounds were nearly the same, the more water soluble cumene had the greatest rate of evaporation.

Crude oil components have a wide range of solubilities in water. McAuliffe (234) studied the effects of chemical structure on the solubility of C_1-C_9 hydrocarbons in fresh water and discovered the following relationships: the solubility of low molecular weight n-paraffins is inversely proportional to their molecular weight; the logarithms of the solubility of the compounds of each homologous series is a linear function of their molar volume; branched chain, saturated and unsaturated hydrocarbons are more soluble than their straight chain counterparts; branched chain substituted cyclic compounds have approximately the same solubility as straight chain substituted cyclic compounds for a given carbon number; ring structures are more soluble than non-ring structures; solubility increases with the degree of unsaturation for a given carbon number; a triple bond is more soluble than two double bonds.

The relatively solubility of oil components determines the predominant components in water soluble extracts of an oil. Boylan and Tripp (44) recovered saturated hydrocarbons, medium and high molecular weight aromatics and polar cyclic compounds from seawater extracts of crude oil. Mackay and Shiu (219) reported that benzene and toluene were predominant in water soluble extracts of crude oil. Medium molecular weight aromatic hydrocarbons, particularly methyl and dimethyl naphthalenes, constituted the greatest portion of water soluble extracts of fuel oils, according to Smith and MacIntyre (307). Lysyj and Russell (218) found that most petroleum products reached equilibrium in water within 24 hours and that the concentration of water soluble components remained stable for several days or weeks, depending on the product tested.

Physical processes play an important role in altering the susceptibility of oil to chemical and biological attack. Oil components may enter a water column as particulates, colloidal suspensions or truly soluble substances. Particulates in water are able to attract hydrophobic substances, such as oil and microorganisms, which may contribute to the dispersion and degradation of oil in water (241). Oil that is not truly water soluble may be accommodated in water as a colloidal suspension. Accommodated oil tends to form oil-in-water emulsions, which increase the surface area available for chemical or biological degradation (82). Formation of oil-in-water emulsions is enhanced by agitation or the presence of surfactants in the water. Colloidal asphaltenes in the oil may act to stabilize oil-in-water emulsions (225). Formation of water-in-oil emulsions decreases the available surface area of oil and slows its chemical and biological degradation considerably (92,268). Water-in-oil emulsions appear as floating masses sometimes referred to as "chocolate mousse."

Weathering may be affected by the chemical reactivity of oil components in water. Eganhouse and Calder (111) found that solute-solute interactions among oil components decreased their water solubility and that increased salinity tended to "salt out" aromatics from aqueous solutions. Gruenfeld (152) increased the recovery of oil from water with organic solvents by increasing the salinity of the water. Formation of oil-in-water emulsions is inhibited by high salt content, low temperature and high pH (82). Certain oil components may mask other components and alter their interaction with the environment (345). Removal or concentration of masking agents by physical processes could potentially change the reactivity of the masked components. The chemical composition of both the oil and the water is important in the weathering process.

Chemical Alterations

Chemical alteration of oil in water is characterized by non-enzymatic changes in the molecular structure of its components. Chemical alterations of hydrocarbons in water occur primarily by substitution and oxidation reactions.

Hydrocarbons are readily substituted by chlorine and other halogens. Halogenated hydrocarbon derivatives have been found in chlorinated wastewater effluents that were not present in the untreated water. Some halogenated hydrocarbons formed in this manner, such as chloroform, may have adverse effects in aquatic environments (337).

Frank (132) stated that virtually all constituents of oil may undergo autooxidation. He defined autooxidation as oxidations that can be brought about by oxygen gas at normal temperatures without the intervention of a visible flame or an electric spark. Oxidation of hydrocarbons by atmospheric oxygen is accomplished through a free radical mechanism as shown below:

1. Initiation $RH \xrightarrow{\text{heat, light}} R\cdot + (H\cdot)$
2. Propagation $R\cdot + O_2 \longrightarrow RO_2\cdot$
   $RO_2\cdot + RH \longrightarrow RO_2H + R\cdot$
3. Termination $R\cdot + H\cdot \longrightarrow RH$
   $RO_2\cdot + \cdot OH \longrightarrow ROH + O_2$
   $R\cdot + R\cdot \longrightarrow R\text{-}R$

Formation of hydrocarbon free-radicals may be catalyzed by heat, light, metal ions, organometallics in oil, mineral salts in seawater or other free-radicals (132). The best known catalysts for liquid phase autooxidation of hydrocarbons are oil soluble derivatives of transition metals, such as naphthenates and stearates of cobalt, manganese, copper, nickel and iron.

After hydrocarbon free-radicals are initiated, oxidation takes place in two distinct phases. In the first phase, the free-radicals interact with molecular oxygen to form peroxides or hydroperoxides. The peroxide intermediates form alcohols, aldehydes, ketones and organic acids in the second phase by additional free-radical or ionic interactions. Once a free-radical is initiated, it may set up a chain reaction that continues to generate new free-radicals. Breakdown of unstable peroxides also produces free-radicals that can either propagate new free-radicals or terminate chain reactions by radical-radical interactions (132).

Initiation of hydrocarbon free-radicals by heat is probably not common in natural waters because of relatively low water temperatures. Photooxidation of oil is common in floating oil slicks, but is limited to the top few centimeters of water. Thin films and emulsions of oil in water are more susceptible to autooxidation than large masses. Oxygen transfer is accelerated in thin films of oil, so it is not a limiting factor (82). Photochemical oxidation of oil is accelerated at wavelengths less than 400 nm, especially in the near ultraviolet and part of the visible spectra (268). Ultraviolet light catalyzed oxidation of olefins has been demonstrated over a temperature range of -50- +150 C (132). Suess (320) reported that significant photooxidation of polynuclear aromatic hydrocarbons occurred in the top layers of the ocean.

Photosensitizers, such as pigments, are capable of enhancing the photooxidation of hydrocarbons. Gesser, et al. (137) tested several photosensitizers and found that xanthone had the most suitable properties for increasing the photooxidation of hexadecane in water. Klein and Pilpel (198) showed that 1-naphthol could act as a photosensitizer in oil. Oil usually becomes more dense and contracts during photooxidation because of polymerization reactions, but 1-naphthol was able to increase the spreading coefficient of oil under certain conditions, possibly by increasing the concentration of water soluble products at the oil-water interface.

The ease with which a hydrogen is removed (abstracted) from a hydrocarbon by a free-radical catalyst determines its susceptibility to free-radical attack.(132). Certain components of oil are much more susceptible to hydrogen abstraction than others. The ease of hydrogen abstraction (single electron transfer) from carbon atoms on the basis of chemical structure is as follows: allylic, benzylic > 3° > 2° > 1° > CH_4 > vinylic. This same order holds true for the ease of formation and stability of free-radicals (250).

Charge delocalization acts to stabilize free-radicals. A resonance stabilized molecule, such as benzene, is extremely resistant to free-radical attack and highly unstable as a free-radical, but a hydrogen attached to the benzylic carbon of a substituted benzene is easily removed by a free-radical catalyst. Substituted benzenes and naphthalenes yield stable hydroperoxide derivatives (163). Cyclohexane, decalin and tetralin also form stable hydroperoxide intermediates (132). Paraffins are more resistant to free-radical attack than olefins due to differences in charge delocalization. The double bond of an olefin is not attacked, but the methylene carbon adjacent to the double bond is highly susceptible to hydrogen abstraction and free-radical formation. Ethers form free-radicals easily and aldehydes are more susceptible than ketones to free-radical formation (163, 357).

Free-radical chain reactions are inhibited by small amounts of phenols, organic amines, organic sulfur compounds and antioxidants (268). These inhibitors act by neutralizing the hydroperoxide radical, $RO_2\cdot$, which stops the propagation of free-radicals. Resonance stabilized free-radicals are formed by the interaction of existing free-radicals with inhibitors, and, since they are highly unstable, they are rapidly inactivated (132). Burwood and Speers (54) noted an increase in the concentration of sulfoxides in the high-boiling, water soluble components of photooxidized oil. They suggested that thiacyclanes in the oil, capable of inactivating free-radicals, reacted with the photooxidized hydrocarbons to form the high-boiling sulfoxides.

Chemical oxidation may act in conjunction with biological oxidation to alter hydrocarbons. According to Zobell (382), the rate of hydrocarbon autooxidation is only 10-50 per cent that of biological oxidation rates, but there is evidence that both types of oxidation occur conjunctively. Roubal and Tappel (287) showed that proteins were polymerized and enzymes were inactivated by free-radical peroxidation of lipids. Hou and Laskin (173) demonstrated that microbial degradation of organic sulfur compounds converted them to more water soluble forms. Biological processes may act to increase or decrease the concentration of inhibitors of free-radical chain reactions in oil. The red-brown color appearing in the aqueous phase during microbial degradation of oil has been identified as 1,2-naphthoquinone. This product is formed by non-enzymatic oxidation of 1,2-dihydroxynaphthalene, a biochemical intermediate of naphthalene degradation by microorganisms (269). Waters (357) described the process by which catechol, a common intermediate of aromatic hydrocarbon degradation, may be oxidized by atmospheric oxygen to a stable, yellow quinone.

Autooxidation is not the only means by which hydrocarbons are oxidized in water. Dagley, et al. (84) found that enzymes extracted from *Pseudomonas* sp. grown on cresol took up one mole of oxygen for each mole of protocatechuic acid oxidized, as expected, but the predicted product, 3-oxoadipate was not formed. The reaction product isolated proved to be 2,4-lutidinic acid, a ring structure containing a nitrogen in the ring. The nitrogen was probably supplied by residual ammonium sulfate in the enzyme preparation, since the enzymes were extracted with ammonium sulfate. The ammonium ion was apparently able to react non-enzymatically with the initial ring fission product of protocatechuic acid to form the new product. This example illustrates how the chemical composition of the aqueous phase can influence the final product formed by hydrocarbon oxidation.

Biological Alterations

Biological alterations of oil are enzyme catalyzed reactions causing molecular changes in the oil components. Although multicellular organisms are able to concentrate and metabolize oil, it is generally too toxic to be extensively degraded by these organisms and the majority of the oil is excreted unchanged. Since microorganisms have a much greater potential for producing chemical changes in oil, this section will deal mainly with microbiological activities that chemically alter oil components.

In 1895, Miyoshi (245) observed the growth of the fungus, Botrytis cinerea, on paraffin. Sohngen (312) and Kaserer (188) discovered that certain bacteria could utilize methane as a carbon and energy source in 1906. By 1913, Sohngen (313) had established that many microorganisms were capable of growth on a wide range of petroleum products. Tausson (325) demonstrated the ability of microorganisms to oxidize crude oil components in 1928. Little interest in the microbial degradation of oil was shown again until 1940, when Mogilevskii (246) proposed that bacteria could be used to prospect for oil and gas deposits. Since that time, a major research effort on various aspects of the microbial degradation of oil has taken place. By 1947, over 100 species of bacteria, yeasts and fungi, in 30 genera, were known to utilize oil components (305). Thousands of microorganisms are now known to utilize petroleum hydrocarbons.

Davis (90) stated that over 50 microbial oxidation reactions of hydrocarbons are known.

The types of microorganisms responsible for oil degradation in water are governed by their ability to survive in aquatic environments. Some factors affecting survival include temperature, pH, nutrient levels, toxic chemicals, predators and antibiotic substances (240, 244). Bacteria are usually predominant in oil enrichment cultures. A number of Gram-positive rods and cocci are known to degrade hydrocarbons in oil, but the majority of oil-degrading bacteria are Gram-negative rods with polar flagella. Rhodes (283) speculated that bacteria of this type have survival advantages in natural waters, but could not explain the reasons. Acidic waters tend to favor the development of yeasts and fungi (332). Obligately psychrophilic bacteria and yeasts are common in polar regions which have cold water temperatures throughout the year (248, 304).

Oil-polluted water has a higher percentage of oil-degrading microorganisms than unpolluted water. Mulkins-Phillips and Stewart (254) enumerated the number of oil-degrading microorganisms in ocean water samples from Halifax to Bermuda and found that most samples contained less than ten per cent oil-degrading microorganisms, but noted an increased percentage of oil-degraders in samples near land, especially in areas previously exposed to oil pollution. Calomiris, et al. (60) found that up to 45 per cent of the bacterial isolates from an estuary were oil-degraders. Hood, et al. (167) obtained poor correlation between hydrocarbon concentration and numbers of oil-degrading microorganisms in water, but found that the ratio of oil-degraders to the total number of microorganisms provided a much better correlation with hydrocarbon concentration.

Some of the more commonly reported oil-degrading microorganisms are listed in Table 2. Fuhs (134) examined the ability of a large number of microorganisms to utilize various petroleum hydrocarbons and showed that the microorganisms exhibited clear preferences for specific hydrocarbon components of petroleum. Most of the microorganisms were able to utilize at least one type of paraffin, while fewer microorganisms were able to grow on aromatic and asphaltic fractions of oil.

Many factors affect the biodegradation of oil in water. The simultaneous availability of water, oil, oxygen and nutrients favors oil degradation by microorganisms (258). Floodgate (126) stated that the rate of microbial degradation of oil varies with the chemical complexity of the oil, the number and type of microorganisms present and the environmental conditions. Horowitz, et al. (169) described some of the factors that enhance the microbial degradation of oil. Their findings are summarized as follows: low molecular weight n-alkanes are most rapidly degraded by microorganisms in the laboratory and in nature; mixed cultures of microorganisms are able

Table 2. Some microbial genera capable of oxidizing hydrocarbons.

Microbial type	Genera	References
Bacteria	*Pseudomonas*	1, 83, 134, 254, 312, 325, 349
	Bacillus	1, 60, 134
	Achromobacter	83, 134, 254
	Alcaligenes	1, 134
	Flavobacterium	1, 22, 134, 254
	Micrococcus	1, 124, 134, 282
	Corynebacterium	1, 60, 83, 134
	Mycococcus	1, 134
	Mycobacterium	1, 134, 196, 346
	Actinomyces	60, 134
	Vibrio	1, 83, 254, 349
	Nocardia	1, 134, 254, 278, 346
	Brevibacterium	1, 22, 83
	Streptomyces	1
	Arthrobacter	1, 83, 199, 281
	Spirillum	282
	Aeromonas	349
	Acinetobacter	124, 349
	Enterobacter	1
	Giberella	1
	Beijerinkia	141, 142
Yeasts	*Candida*	1, 134, 169, 242, 346
	Monilia	134
	Micromonospora	134
	Endomyces	134
	Debaromyces	134, 242
	Hansenula	134
	Pichia	1, 242
	Torulopsis	1, 242
	Hormodendrum	1
	Rhodotorula	242
	Trichosporon	242
	Rhodosporidium	242
	Acremonium	134
	Mycotorula	4
Fungi	*Penicillium*	1, 134, 325
	Citromyces	134
	Scopulariopsis	134
	Sporotrichium	1
	Rhizopus	1
	Cladosporium	1, 348, 349
	Botrytis	245
	Mucor	165
	Aspergillus	1, 134, 325
	Pullularia	349

to degrade more oil than pure cultures; the ability of microorganisms to degrade oil in water is dependent on the presence of nitrogen and phosphorus.

The composition and concentration of oil in water has a major effect on its biodegradation. Walker, et al. (354) found that crude oil was more easily degraded by microorganisms than fuel oil and suggested that the high concentration of high molecular weight aromatic hydrocarbons in fuel oil decreased the rate of biodegradation. Preferential use of saturated hydrocarbons in south Louisiana crude oil by microorganisms was reported by Kator, et al. (189). They observed a consistent loss of saturates and an increase in polar compounds after microbial degradation. Diauxic growth appeared to occur with rapid initial growth on paraffins up to C_{18} followed by a lag phase and a second growth phase on high molecular weight paraffins. Wuhrmann (369) observed that diauxic growth occurs only on substrates metabolized by enzymes having the same precursors. He found little evidence for diauxic growth of microorganisms on complex substrates and suggested a multiphasic attack as a more likely alternative.

Adaptation experiments by Fredericks (133) showed that microorganisms grown on relatively recalcitrant hydrocarbons adapted readily to growth on more easily degraded hydrocarbons, but the reverse adaptation was much more difficult. He ranked petroleum hydrocarbons according to their relative ease of biodegradation as follows: n-paraffins(C_8-C_{18}) > branched chain alkanes > n-paraffins (C_5-C_8) > cyclic hydrocarbons.

Tagger, et al. (323) observed that the amount of hydrocarbon degradation by microorganisms increased as the substrate concentration was increased up to a limiting value of 800 mg/l. Some of the hydrocarbon or non-hydrocarbon components of oil may exert bacteriostatic or bacteriocidal effects as the concentration is increased (383). According to Ahearn, et al. (3), south Louisiana crude oil inhibited yeast growth at concentrations above four per cent in the growth medium.

Accumulation of metabolic products during microbial degradation of oil may limit biodegradation by toxic or catabolite repression effects. Intermediate products of oil do not usually accumulate in the presence of mixed cultures of microorganisms, but do affect pure cultures (382). Atlas and Bartha (24) identified fatty acids as feedback inhibitors of oil degradation by pure cultures of bacteria. Bartha and Atlas (30) noted that salicylates formed during naphthalene degradation inhibited further utilization of naphthalene by *Pseudomonas* sp.

The solubility of petroleum components in aqueous environments affects the biodegradability of oil. Several models have been proposed to explain microbial growth at oil-water interfaces (67, 69, 105). These models predict exponential growth rates as long as the concentration of dissolved hydrocarbon is not limiting and the solubilization rate is faster than the rate of utilization. If surface area is a limiting factor, a linear growth rate is predicted. Wodzinski, et al. (366, 367, 368) determined that the consumption rates and generation times of Pseudomonas sp. growing on solid hydrocarbon substrates were dependent on the amount of dissolved substrate rather than the amount of solid substrate. Generation times on aromatic hydrocarbon substrates were related to the water solubility of the substrates.

Shifts in the composition of oil occur during microbial degradation as oil components are oxidized to new products. Polar, nonhydrocarbon NSO's and asphaltenes are formed from hydrocarbon components, resulting in the formation of a low wax, high specific gravity residue (376). Jobson, et al. (184) reported a change in the specific gravity of crude oil from 0.827 to 1.046 after 21 days incubation with microorganisms at 30 C. Cook and Westlake (78) analyzed the composition of northern crude oils before and after microbial degradation. The results they obtained with Prudhoe Bay crude oil are shown in Table 3. The saturate and aromatic fractions were decreased by biodegradation, but the NSO and asphaltene fractions were increased. The polar asphaltenes and NSO's consist of phenolic acids, carboxylic acids, ketones, esters, pyridines, quinolines, carbazoles, sulfoxides, amides, porphyrins, etc. (350).

Table 3. Prudhoe Bay crude oil composition before and after microbiological degradation (78).

Crude oil fraction	% Composition	
	Control	Degraded
Soluble asphaltenes	5.68	4.72
Insoluble asphaltenes	1.57	8.82
Saturates	38.70	28.79
Aromatics	36.40	30.49
Soluble NSO's	12.80	15.22
Insoluble NSO's	5.94	11.59

NSO - Nitrogen, sulfur and oxygen containing compounds.

The types and relative numbers of oil-degrading microorganisms present in water affects the biodegradation of oil in water. Kator, et al. (189) discovered that oil slicks exposed to oil-degrading microorganisms disappeared twice as fast as those subjected to evaporation, only. Mixed cultures of microorganisms have been shown to degrade more oil at a faster rate than pure cultures (281, 386). Ahearn, et al. (3) found that mixed yeast cultures could grow on hydrocarbon substrates that would not support the growth of the yeasts in pure culture. Sequential growth of bacteria on crude oil in pure and mixed culture was demonstrated by Horowitz, et al. (169). The biodegradation products of one bacterial strain acted as the growth substrate for the next strain. Four bacterial strains were capable of converting 75 per cent of the crude oil to a solvent insoluble form in this manner. Walker and Colwell (350) reported distinct differences in the biodegradation of petroleum by microorganisms from oil-polluted and oil-free sediments obtained from Chesapeake Bay (Table 4). Chronic input of oil into water allows microorganisms to adapt to growth on oil and increases the relative number of oil-degraders (30).

Fair, et al. (116) described the process of benthal decomposition in polluted sediments as occurring in three stages: 1) intensive fermentation, 2) gradual consolidation and 3) quiescent stabilization. A rapid development of predominantly bacterial species and a drop in

Table 4. Comparison of petroleum biodegradation by microorganisms from oil-polluted and oil-free sediments of Chesapeake Bay after incubation for 10 days at 19 C (350).

Measured parameter	Oil-polluted sediment	Oil-free sediment
Heterotrophic bacteria	3×10^8/ml	8×10^5/ml
pH change	7.3 to 6.6	7.3 to 7.1
% oil degraded	50	16
Predominant isolates	*Pseudomonas* *Vibrio* *Acinetobacter*	*Pseudomonas* *Corynebacterium*
Intermediate products	Low concn. *n*-pentane insoluble asphaltenes Carboxylic acids	High concn. *n*-pentane insoluble asphaltenes Neutral compounds Esters

pH was observed in the first stage. The pH recovered in the second stage and a varied group of protozoans developed along with a highly specialized group of bacteria capable of degrading a wide range of compounds. Bacterial numbers decreased in the third stage, and worms and other aquatic organisms honeycombed the sediments, increasing their surface area and oxygen content. Similar events have been reported during the microbial degradation of oil in water (281, 350).

A shift in predominant bacterial types was noted by Hood, et al, (167) in oil-polluted versus oil-free marsh sediments. The ratio of chromogenic bacteria to total bacteria and the relative number of cellulolytic bacteria decreased in a marsh polluted with crude oil. Walker and Colwell (353) found that the oil-degradation potential of microorganisms obtained from the water column was greater than that of microorganisms obtained from the sediment. Shortly after an oil spill, bacterial numbers increase rapidly, followed by later development of yeasts and fungi. Ciliate populations also increase after an oil spill and graze on the bacteria and yeasts on the surface of emulsified oil droplets (332). Walker and Colwell (349) observed aggregates of bacteria, yeasts and fungi in association with oil droplets after microbial degradation. They also found that bacterial numbers oscillated after reaching stationary phase, possibly due to the predatory action of ciliates.

Microorganisms tend to concentrate at oil-water interfaces, so that the number of microorganisms at an interface may be 100-1000 times greater than in the surrounding water (189, 376). Marshall and Cruickshank (230) isolated microorganisms that oriented perpendicularly to air-water, oil-water and solid-water interfaces and suggested that the cells were more hydrophobic at one end than the other. Marshall (229) stated that microorganisms have predominantly hydrophobic surfaces and are attracted to non-aqueous phases. Particulates in water attract microorganisms and organic molecules and may influence the degradation of oil in water.

The hydrophobic properties of microorganisms vary considerably and may be a determining factor in their ability to degrade hydrophobic substrates. Acid-fast bacteria can enter or leave oil phases freely, while most bacteria remain at the oil-water interface (229). Kjelleberg (197) studied the ability of bacteria to penetrate a lipid monolayer of oleic acid on water by measuring changes in the surface pressure of the lipid. *Acholeplasma laidlawii*, a mycoplasma with an exposed cytoplasmic membrane and no cell wall, was able to penetrate the lipid monolayer readily. *Pseudomonas fluorescens* increased the viscosity of the lipid layer, but was unable to penetrate it. *Serratia marinorubra* was able to enter the lipid layer only after the pH was increased to 10.5, probably because of ionization of the oleic acid.

A number of oil-degrading microorganisms, such as *Corynebacterium hydrocarbonoclastus* (377), *Arthrobacter* sp. (281), *Acinetobacter* sp. (124) and yeasts and fungi (378), are capable of emulsifying oil in pure or mixed culture. Zajic (379) observed that the paraffin fraction of Bunker C fuel oil was degraded exclusively by a bacterial culture with emulsifying properties. He purified an emulsifying agent from the culture and identified it as a high molecular weight polysaccharide. The emulsifier was apparently biosynthesized from the paraffin fraction of the oil. Microbial emulsification of oil increases its surface area and rate of biodegradation in water (281).

The ability of microorganisms to sequester hydrocarbons in their cytoplasm was demonstrated by Finnerty, et al. (123, 124). Transmission electron microscopy revealed the presence of inclusions in hydrocarbon-grown bacterial cells that were absent in bacteria grown on non-hydrocarbon substrates. The hydrocarbons in the inclusions were apparently unchanged, indicating that an active transport mechanism for hydrocarbons is present in oil-degrading bacteria. Cells in contact with oil droplets or tar balls had a high number of hydrocarbon inclusions. The bioconcentrated oil may be excreted at a later time as metabolic products.

Temperature is a major factor limiting the degradation of oil by microorganisms in natural waters, since over 90 per cent of the water in the ocean is 5 C or colder (248). Ingraham and Stokes (179) stated that psychrophilic bacteria are able to carry out nearly the same metabolic activities as mesophilic bacteria, but at a slower rate. Some commonly isolated genera of psychrophilic microorganisms include *Achromobacter*, *Alcaligenes*, *Proteus*, *Pseudomonas*, *Serratia*, *Vibrio*, *Bacillus*, *Micrococcus*, *Candida* and *Torulopsis* (117, 248, 304). Sinclair and Stokes (304) isolated a greater number of psychrophilic yeasts than bacteria in the Antarctic. Gram-negative, rod-shaped bacteria are isolated most often at psychrophilic temperatures. Bacteria of this type survive well at extremely cold temperatures and many are able to degrade oil (118).

Westlake, et al. (359) noted differences in the oil-degrading ability of microorganisms at mesophilic and psychrophilic temperatures. Some oil components that were readily degraded at 30 C resisted degradation at 4 C. Walker and Colwell (349) found that bacterial degradation of oil was predominant from 0-5 C and that yeast growth became significant at 10 C. Atlas and Bartha (21) reported that maximum hydrocarbon utilization by microorganisms occurs between 25 and 37 C. They showed that the amount of oil degraded by microorganisms was temperature dependent, accounting for an oil loss of 30 per cent at 2 C, 40 per cent at 18 C and 50 per cent at 30 C after eight weeks. Mulkins-Phillips and Stewart (255) found that a 10 C decrease in temperature caused a two-fold increase in the generation time of bacteria growing on oil. Atlas (19) observed a greater loss of paraffins from crude oil at 10 C than at 20 C and suggested that the most easily degraded hydrocarbons were preferentially utilized at low temperatures.

Both the enrichment and growth temperature affect the generic composition of microorganisms grown on oil. Microorganisms grown on oil at psychrophilic temperatures readily metabolized oil at mesophilic temperatures, but those adapted at mesophilic temperatures grew poorly at psychrophilic temperatures (376). Atlas and Bartha (21) found that the microorganisms in winter water samples were able to degrade more oil at 5 C than microorganisms in summer water samples.

Microbial degradation of oil is known to occur at subzero temperatures. Zobell (385, 386) detected oil degradation by microorganisms in slush ice at -1.1 C. A higher percentage of oil was converted into biomass at -1.1 C than at 8 or 25 C. Growth rates were nearly the same at -1.1 and 4 C. Button (58) detected the release of $^{14}CO_2$ from ^{14}C-dodecane at -1.7 C in the Arctic Ocean. Atlas (20) was unable to demonstrate significant degradation of oil under ice, however. Bunch and Harland (52) showed that the indigenous microorganisms in the Beaufort Sea were able to oxidize petroleum hydrocarbons at natural water temperatures. No change in the concentration of aliphatic hydrocarbons was evident for four weeks, but nearly complete degradation of the aliphatics had occurred in 12 weeks at 5 C.

Farrell and Rose (117) reported that certain enzymes, such as lipase in Pseudomonas fragi, were synthesized at low temperatures but not at high temperatures. They suggested that differences in the minimum growth temperatures of mesophiles and psychrophiles may be determined by their ability to transport molecules across membranes at low temperatures.

Seasonal selection of microorganisms by water temperature may influence the biodegradation of oil in natural waters. Sieburth (302) noted a shift in the predominance of mesophiles and psychrophiles at approximately 12 C. Hydrocarbon-degrading mesophiles and psychrophiles were present in sediments in the winter, but the psychrophiles disappeared from the sediments in the summer. Seasonal selection of microorganisms by water temperature appeared to be uniform in all taxonomic groups and did not favor specific genera. Nedwell and Floodgate (260) detected little difference in the total number of bacteria in water at different temperatures, but found significant shifts in the relative percentages of psychrophiles, mesophiles and thermophiles with seasonal changes in water temperature. Ezura, et al. (115) followed changes in the predominant bacteria in the waters of a bay throughout a year and observed an increase in psychrophilic bacteria at temperatures below 5 C. Pseudomonas, *Achromobacter* and *Flavobacterium* constituted over 80 per cent of the isolates from the bay. *Vibrio* spp. were predominant over *Flavobacterium* spp. at low temperatures.

Low levels of non-hydrocarbon nutrients, especially nitrogen and phosphorus, have a limiting effect on the biodegradation of oil in natural waters. Atlas and Bartha (23) reported that biodegradation of crude oil in natural seawater amounted to only three per cent after 18 days at 28 C. Addition of nitrogen or phosphorus supplements to the seawater separately had little effect, but addition of both supplements increased oil biodegradation to 70 per cent. Gordon (146) showed that both nitrogen and phosphorus limited dissolved oxygen depletion in polluted subarctic waters. Addition of inorganic nitrogen and phosphorus to the waters increased dissolved oxygen depletion significantly, but only slight increases were noted when nitrogen and phosphorus were added separately.

Limiting concentrations of nitrogen and phosphorus in water are rapidly assimilated into microbial biomass and are unavailable for utilization by other microorganisms (369). Bushnell and Haas (56) established a minimum requirement of 50 mg/l phosphate for growth of *Pseudomonas* on kerosine, but did not find nitrogen or sulfur limiting. Mulkins-Phillips and Stewart (255) also reported that microbial growth on hydrocarbons was stimulated by phosphorus, but not by nitrogen. Zajic and Dauglis (376) determined that 10 mM nitrate and 0.35 mM phosphate concentrations were optimal for mixed culture utilization of petroleum hydrocarbons. The non-hydrocarbon nutrient requirements for oil degradation in natural waters varies with the amount of nutrients already present in the waters.

Trace elements and growth factors may be important in the biodegradation of oil. Meyers and Ahearn (242) found that yeasts grew faster on oil in natural seawater medium than in artificial salts medium, indicating that the seawater provided essential growth factors. Dibble and Bartha (97) stimulated oil biodegradation in iron deficient water by adding an organic iron supplement.

Aeration has been shown to increase the microbial utilization of oil (184). Rashid (277) observed that Bunker C fuel oil disappeared most rapidly from ocean sediments along shorelines with strong wave action and showed that both physical and microbial oxidation processes were enhanced by wave action. Shelton (297) reported a greater loss of hydrocarbons under anaerobic conditions than under aerobic conditions. He was unable to correlate hydrocarbon loss with sulfate reduction, however, and suggested that the water soluble intermediates formed under anaerobic conditions were not completely recovered. The value of aeration in the biodegradation of oil has been confirmed by others (49, 184, 298). Zobell (383) estimated that the complete oxidation of one gallon of crude oil would require all of the dissolved oxygen in 320,000 gallons of seawater. He determined that three to four moles of oxygen were consumed for each mole of mineral oil degraded.

The salinity of water may affect the hydrocarbon degrading activities of some microorganisms. According to Tagger, et al. (323), seawater diluted by river water had less oil-degrading potential than undiluted seawater, probably because the freshwater microorganisms were not adapted to saline conditions and diluted the number of saline-adapted microorganisms capable of growing on oil.

The pH of water influences the development of hydrocarbon-degrading microorganisms. Bushnell and Haas (56) showed that the growth of Pseudomonas on kerosine was not adversely affected over a pH range from approximately 6.5-9.0. Bacterial growth on petroleum generally causes a drop in pH, encouraging the development of yeasts and fungi (332, 350). Atlas (18) isolated a greater number of fungi than bacteria from arctic waters chronically polluted by oil seeps. The relatively low pH of these waters apparently had a selective effect for the fungi. Alteration of environmental conditions by microorganisms may be a significant factor controlling the succession of microbial populations during oil biodegradation.

Kim and Zobell (195) reported that cell-free enzymes influenced the degradation of pollutants in the ocean. They discovered that the total enzyme activity in ocean water was greater than the enzyme activity of its biomass. Seasonal changes in the predominant organisms in the ocean water influenced the type and concentration of cell-free enzymes in the water.

Microbiological Degradation Intermediates of Oil

A number of general reviews of the biochemical pathways of hydrocarbon utilization by microorganisms are available (103, 128, 177, 331, 339), as well as more specific reviews of pathways, such as aliphatic hydrocarbon oxidation (375), cycloparaffin oxidation (266) and aromatic hydrocarbon oxidation (139). An overview of the early steps of hydrocarbon degradation and the enzymes involved is presented to show what products are normally produced from petroleum hydrocarbons by microorganisms and to provide a basis for determining the origin of toxic and/or mutagenic intermediates produced in hydrocarbon substrates by microorganisms. The biodegradation products of alkanes, olefins, cycloalkanes, cycloalkenes and aromatics will be discussed.

Alkanes

The alkane fraction of petroleum is most easily biodegraded and is capable of supplying both carbon and energy for microbial growth. Table 5 shows that most of the cellular components of microorganisms can be biosynthesized from alkanes. Some alkanes

Table 5. Microbial metabolites of petroleum hydrocarbon substrates (1).

Substrates			
Alkanes	Aromatics	Polynuclear aromatics	Terpenes cycloalkanes cycloalkenes
Fatty acids	Fatty acids	Substituted acids	Fatty acids
Alcohols	Substituted acids	Diols	Alcohols
Aldehydes	Substituted alcohols	Dihydrodiols	Diols
Ketones	Diols	Diones	Ketones
Epoxides	Phenols	Epoxides	Aldehydes
Hydroperoxides	Hydroperoxides	Phenols	
Esters	Epoxides	Ring cleavage intermediates	
Glycolipids	Substituted alkenes		
Alkenes	Ring cleavage intermediates		
Amino acids	TCA cycle intermediates		
TCA cycle intermediates	Vitamins		
Nucleic acids	Antibiotics		
Vitamins	Aldehydes		
Pigments			
Sugars			
Polysaccharides			
Enzymes			
Dipeptides			

act as inducers for the synthesis of paraffin-oxidizing enzymes in microorganisms (341).

The main pathway of alkane biodegradation is by monoterminal oxidation of a methyl group with successive formation of the corresponding primary alcohol, aldehyde and monoic acid intermediates. The resulting carboxylic acid is further degraded by beta-oxidation (339). Alkanes may also be oxidized at subterminal and diterminal positions, but these are considered minor pathways.

Hydroperoxides, mixed function oxidases or NAD(P)-linked dehydrogenases may participate in the initial oxidation reactions of alkanes. Oxidation of terminal methyl groups is accomplished by an enzyme complex consisting of an NAD(P)-linked dehydrogenase, a mixed function oxidase and an electron carrier system.

Subterminal oxidation of alkanes may proceed via mixed function oxidases or hydroperoxide intermediates. A mechanism involving the production of an alkyl hydroperoxide intermediate has been proposed to explain the formation of methyl ketones from saturated hydrocarbons, but it appears to be limited to n-alkanes with less than ten carbons (187). Oxidation of subterminal carbons by mixed function oxidases requires an enzyme complex consisting of a methylene hydroxylase, a flavoprotein and cytochrome P-450. Methyl ketones formed by methylene group oxidation may be further degraded to secondary alcohols or may enter the beta-oxidation pathway via an ester intermediate which is cleaved to yield acetate and a primary alcohol (127). Klein, et al. (199) showed that subterminal oxidation of alkanes could occur at several sites on the carbon chain. They found that an *Arthrobacter* sp. grown on an auxiliary carbon source in the presence of *n*-hexadecane produced a series of monohexadecanones in the growth medium. No alcohol or aldehyde intermediates were detected in the medium and an inverse relationship between the concentration of ketone product formed and the distance of the oxidized carbon from the terminal carbon was observed.

Omega-oxidation of monoterminally-oxidized alkanes results in the formation of dicarboxylic acids. The enzyme system required for *omega*-oxidation consists of a DPN-reductase, rubredoxin and an *omega*-hydroxylase (274, 275). Cytochrome c accepts electrons from rubredoxin and the *omega*-alcohol, aldehyde and carboxylic acid intermediates are formed as in monoterminal oxidation. The resulting dicarboxylic acid may undergo *beta*-oxidation from either end.

Yeasts (201, 314) and fungi (165, 166) utilize biodegradation pathways similar to those of bacteria, as the same intermediate products are formed.

Microorganisms growing on alkanes as a sole carbon source incorporate fatty acid intermediates into their cellular lipids. Bacterial lipids may consist of branched or straight chain saturated and monounsaturated fatty acids and cyclopropane fatty acids (211). Polyunsaturated fatty acids and sterols are not present in bacterial lipids, but sterols are present in some fungi. The unsaturated fatty acid content of bacteria growing on alkanes is significant, indicating the ability of microorganisms to unsaturate alkanes (223). King and Perry (196) used radiolabelling techniques to show that long chain saturated fatty acids were unsaturated by Mycobacterium vaccae. Souw, et al. (314) found that myristic acid could be directly unsaturated to tetradecenoic acid by a Candida sp.

The cellular concentration of fatty acids, amino acids, diaminopimelic acid and glucosamine was similar in bacteria grown on glucose or alkanes, but only even chain length fatty acids were found in bacteria grown on glucose (346). Bacteria grown on even-numbered alkanes from C_{12}-C_{18} incorporated only even-numbered fatty acids of the same chain length as the substrate into their lipid, while bacteria grown on odd-numbered alkanes from C_{11}-C_{17} incorporated both even and odd-numbered fatty acids.

Branched chain alkanes are more resistant to oxidation by microorganisms than straight chain alkanes because of steric hindrances to enzyme activity. Thijsse and van der Linden (327) showed that 2-methylhexane could be oxidized at the C_1 or C_6 positions to yield 2-methylhexanoic acid and 5-methylhexanoic acid, respectively. The 2-methylhexanoic acid was cleaved to form propionic and butyric acids and the 5-methylhexanoic acid was cleaved to form isovaleric and acetic acids. Isovaleric and propionic acids are not *beta*-oxidized and probably undergo a CO_2 fixation step before they are further degraded.

Co-oxidation of alkanes to acids, alcohols, ketones and aldehydes has also been reported (339). The enzymes responsible for this type of oxidation have not been well characterized, but are thought to be oxygenases.

Olefins

Olefins are not usually present in crude oil, but may appear after catalytic cracking of petroleum (225) or after microbial degradation of alkanes (223). The major pathway of olefin biodegradation is by terminal methyl group oxidation as in alkane oxidation. Oxidation at a terminal double bond, resulting in the formation of a 1,2-epoxide, represents a minor degradation pathway. May and Abbott (233) reported that the *omega*-hydroxylation system of *Pseudomonas oleovorans* could also catalyze the epoxidation of terminal double bonds of olefins. Octane-grown cells of *Pseudomonas oleovorans* formed 1,2-epoxyoctane and 7-octene-1-ol from 1-octane and 1,2-7,8-diepoxyoctane from 7,8-epoxy-1-octene (233,293). No evidence of methyl ketone or secondary alcohol formation was found in the system. Epoxidation of terminal olefins is analogous to the epoxidation of unsaturated steroids by *Nocardia* and fungi.

Candida lipolytica has been shown to incorporate $^{18}O_2$ into terminal olefins to form diols (180). Enzymatic dihydroxylation is usually difficult to prove conclusively, since it is easily masked by non-enzymatic hydrolysis of epoxides (178), but the presence of labelled oxygen in both hydroxyl groups showed that both atoms of molecular oxygen were incorporated into the olefins. Candida lypolytica was also able to oxidize olefins at the terminal methyl group.

Microbial degradation of olefins apparently does not result in the accumulation of initial oxidation products, since unsaturated fatty acids of the same chain length as the parent olefin are not incorporated into microbial lipid and the fatty acids derived from alkenes are usually saturated (224). The alkene degradation pathway proposed by Huybregtse and van der Linden (178) suggests that omega-unsaturated fatty acids of the same chain length as the olefins are initially formed by methyl group oxidation. The unsaturated fatty acids are saturated by epoxidation and diol formation followed by successive formation of alpha hydroxy acids, aldehydes and saturated fatty acids of shorter chain length, according to the proposed pathway.

Long chain esters were recovered from cultures of bacteria growing on 1-alkenes by Stewart, et al. (317). Micrococcus certificans formed 15-hexadecenyl palmitate from 1-hexadecene and a complex mixture of esters from 1-octadecene. Ester formation proceeds via terminal methyl group oxidation, as the olefin double bond is intact in the ester intermediates.

Hydroperoxides may be involved in the initial oxidation of olefins by microorganisms. It is suspected that certain enzymes may act as carriers of singlet oxygen, since soybean lipoxygenase forms the same hydroperoxide intermediate from linoleic acid as that produced by non-enzymatic addition of singlet oxygen (71, 139, 191). It is possible that some of the olefin intermediates could be formed by hydroperoxide breakdown, but there is no direct evidence for the existence of singlet oxygen carrying enzymes in microorganisms.

Cycloalkanes and Cycloalkenes

Cycloalkanes and cycloalkenes are not as readily utilized by microorganisms as sole carbon sources as their straight chain counterparts. Ooyama and Foster (266) showed that C_3-C_8 cycloparaffins did not support the growth of a soil-pseudomonad-like bacterium, but were oxidized by co-metabolism. Beam and Perry (32) studied the degradation of ^{14}C-labelled cycloparaffins by microorganisms isolated from marine mud. Although none of the isolates could utilize the cycloparaffins in pure culture, several isolates co-oxidized them to ketone intermediates which were degraded by other isolates. They suggested that degradation of cycloparaffins by mixed cultures is accomplished by a combination of co-metabolism and commensalism.

An oxygenase is thought to be involved in the co-metabolism of cyclic hydrocarbons. Co-metabolism of recalcitrant molecules is enhanced by growth substrates requiring an oxygenase for metabolism. Biodegradable analogs of the molecule to be co-metabolized are often used for this purpose (32). Horvath (172) reviewed the literature on co-metabolism and concluded that many recalcitrant organic compounds are biodegraded in nature by co-oxidation reactions of the naturally-occurring microorganisms.

Cyclic ketones and alcohols are common products of cycloparaffin co-metabolism (280). Cyclohexanone and cyclohexanol are produced from cyclohexane (1). Cycloparaffin rings are usually not cleaved by co-metabolism, but this may occur in a few instances. Cyclopropane is cleaved by several microorganisms to form propionaldehyde (1,266). Decalin is cleaved to form adipic or pimelic acid by Flavobacterium sp. and cyclohexane is cleaved to form adipic and valeric acids by Pseudomonas aeruginosa when these substrates are the sole carbon source (1). Alkyl substituted cycloparaffins may be oxidized to cyclic acids by co-metabolism (91). Two carbon alkyl substituents are resistant to co-oxidation, but one and three carbon substituents are readily co-metabolized to acids. Cycloparaffins substituted with omega fatty acids longer than four carbons are able to support microbial growth by beta-oxidation.

Microbial oxidation products of cycloalkenes include ketones, epoxides, alcohols and diols (1). Cycloalkenes, like cycloalkanes, usually require co-metabolism to be degraded and an auxiliary growth substrate is generally needed for microorganisms to co-oxidize cycloalkenes. Fungi are more active in co-metabolism of cycloolefins than bacteria. Terpene hydrocarbons are co-oxidized in a manner similar to cycloalkenes.

Aromatics

The primary step in the microbial oxidation of aromatic hydrocarbons is ring hydroxylation. Gibson (140) used $^{18}O_2$ to show that both atoms of molecular oxygen were enzymatically incorporated into benzene by *Pseudomonas putida*. The initial oxidation product formed by microorganisms growing on aromatic hydrocarbon substrates has not been isolated, but a cyclic hydroperoxide intermediate has been proposed to explain the formation of subsequent intermediates. Catechol and 3, 5-cyclohexadiene-1, 2-diol were readily identified as intermediates in the microbial oxidation of benzene by *Pseudomonas aeruginosa*. Mammalian enzymes incorporate only one atom of molecular oxygen into aromatic rings to form epoxide (arene oxide) intermediates and obtain a second oxygen atom from water to form diol intermediates.

NAD-dependent dioxygenases catalyze the dihydroxylation of aromatic rings. Catechol-1, 2-dioxygenase catalyzes the incorporation of molecular oxygen into benzene to form catechol. Substitution of a single oxygen atom into benzene to form phenol or into phenol to form catechol is catalyzed by a monooxygenase (103, 156). Depending on the starting substrate, the biochemical pathways of aromatic hydrocarbon degradation converge upon one of three dihydroxydiol intermediates prior to ring cleavage, namely, catechol, protocatechuate or gentisate (103, 138).

The next major step in the catabolism of aromatic hydrocarbons is ring cleavage which requires dioxygenases specific for certain substrates. Pyrocatechase and metapyrocatechase are specific for catechol, protocatechase-3, 4-dioxygenase is specific for protocatechuic acid and gentisate-1, 2-dioxygenase is specific for gentisic acid. Carboxylic acids are formed as a result of ring fission. Pyrocatechase splits catechol at the ortho position between hydroxyl groups to form *cis, cis*-muconic acid, while metapyrocatechase splits catechol at the meta position adjacent to one of the hydroxyl groups to form *alpha*-hydroxymuconic semialdehyde (156, 157).

After ring fission, the *ortho* fission product of catechol is further degraded to muconolactone, *beta*-ketoadipate and citric acid cycle intermediates, successively. The *meta* fission product of catechol forms 2-oxopent-4-enoate, 4-hydroxy-2-oxovalerate, pyruvate and acetaldehyde intermediates in succession. Protocatechuic acid forms *beta*-carboxy-*cis, cis*-muconate, keto and enol lactones, *beta*-ketoadipate and citric acid cycle intermediates. Gentisic acid forms maleylpyruvate upon cleavage which is split to form pyruvate and either malate or fumarate. Substituted aromatics are degraded by pathways similar to catechol, but different final products are formed. *Meta* cleavage appears to occur mainly with alkyl substituted derivates of catechol, while *ortho* cleavage is restricted to catechol and its precursors (103).

Co-oxidation is an important mechanism in the degradation of alkyl substituted aromatic rings. Raymond, et al. (278) reported the formation of *o*- and *p*-toluic acids from *o*- and *p*-xylenes by *Nocardia*. Some common co-oxidation products of short chain alkyl substituents of benzene are benzoic acid, phenylacetic acid and phenylacrylic acid (1). Co-oxidation does not result in aromatic ring fission, but the position and type of functional group produced by co-metabolism affects the further biodegradability of substituted rings (70). Methyl naphthalenes substituted in the *beta* position were co-oxidized to carboxylic acids, but methyl groups in other positions were not oxidized (278).

Naphthalene is oxidized to *cis*-1,2-dihydro-1,2-dihydroxynaphthalene by a dioxygenase (256). The above reaction product was originally thought to be in the *trans* configuration (355), similar to mammalian enzyme reactions, but was later corrected to the *cis* configuration (64, 183), which is consistent with microbial enzyme reactions. Microorganisms degrade *cis* faster than *trans* isomers and convert *trans* to *cis* configurations before degrading some compounds (64). It has been established that mammals utilize monooxygenases to form arene oxides from aromatic substrates and, subsequently, *trans*-diols. Bacteria utilize dioxygenases to produce *cis*-diols, presumably via a dioxetane intermediate (142).

The *cis*-dihydrodiol of naphthalene is dehydrogenated to form 1,2-dihydroxynaphthalene. This intermediate may be enzymatically degraded to salicylic acid by removal of pyruvic acid from the hydroxylated ring and further degraded to catechol or it may undergo non-enzymatic oxidation to 1,2-naphthoquinone, especially in media deficient in Mg^{++} and Fe^{+++} or at an alkaline pH. The characteristic red-brown to orange color in the basal medium after microbial degradation of naphthalene is caused by the formation of 1,2-naphthoquinone (256,269).

Polynuclear aromatic hydrocarbons are more resistant to microbial degradation than mono- or di-nuclear aromatics and their rate of transformation is relatively slow (162). A *cis*-dihydrodiol is usually the first identifiable intermediate, similar to the mono- and di-aromatics. The *cis*-dihydrodiol is dehydrogenated to the *cis*-diol. Ring fission results in the formation of salicylate which is degraded to catechol. Two or more rings must be split to form the salicylate intermediate and a series of carboxylic acid intermediates are formed in the process (138).

Napthalene is always hydroxylated at the 1,2 position by microbial enzymes, but polynuclear aromatic hydrocarbons may be hydroxylated at more than one position (193). Electron dense K- and L-regions of polynuclear aromatics affect the site of enzyme attachment and ring fission (284). The 9, 10 position of anthracene is an L-region and the 9, 10 position of phenanthrene is a K-region. Enzyme attachment and ring cleavage occur on the same ring of the linear polynuclear aromatic, anthracene, and on separate rings of the angular polynuclear aromatic, phenanthrene. For this reason, anthracene is hydroxylated at the 1, 2 position and phenanthrene is hydroxylated at the 3, 4 position by microbial enzymes. Methyl substitution at the C_9 position of phenanthrene decreases its rate of oxidation due to partial blocking of the enzyme.

Inducible enzymes play a major role in the early steps of hydrocarbon oxidation by microorganisms. There is also evidence that constitutive enzymes are active in the early steps of hydrocarbon degradation. Evans, et al. (114) suggested the Fe^{++}-dependent oxygenase catalyzing *ortho* cleavage of dihydrodiols was constitutive in *Pseudomonas* spp. The role of constitutive enzymes in the early steps of hydrocarbon degradation is not as well defined as for later steps.

A number of studies have shown the importance of inducible enzymes for the degradation of specific hydrocarbon substrates. Barnsley (29) found that enzymes extracted from *Pseudomonas* grown on naphthalene readily oxidized fluoranthene, but not benzo(a)pyrene.

Sparnins and Dagley (315) showed that unusual substrates, such as gallic and syringic acids, induced *meta* fission dioxygenases in *Pseudomonas*. In the absence of these inducers, protochatechuate-3,4-dioxygenase was formed to split protochatechuic acid by *ortho* fission. The unusual substrates acted as competitive inhibitors of protochatechuate oxidation. Chakrabarty (65) induced *meta* fission oxygenases in bacteria with salicylate, but not with catechol. Cytochrome *P*-450$_{cam}$ is induced in *Pseudomonas putida* by camphor and is required for the degradation of camphor.

Extrachromosomal plasmids are involved in the degradation of some hydrocarbons. Chakrabarty (65) and Dunn and Gunsalus (106) reported the existence of gene clusters in *Pseudomonas* that were specific for the degradation of salicylate (SAL plasmid) and naphthalene (Nah$^+$), respectively. The gene clusters were conjugable, curable with mitomycin C and spontaneously lost from the bacteria, indicating their extrachromosomal nature. The plasmids coded for enzymes active in the early degradation steps of the aromatic substrates. There are also plasmids in *Pseudomonas* spp. for the degradation for toluene (TOL plasmid) and octane (OCT plasmid) (66, 68).

Toxic Effects of Oil in Aquatic Environments

Petroleum hydrocarbons and non-hydrocarbons may exhibit acute or chronic toxicity in aquatic environments. Wilbur (360) described the difference between the acute and chronic toxicity of pollutants to aquatic organisms as follows:

> Ordinarily, if death or disability occurs within 96 hours or less, acute toxicity is concluded. Chronic toxicity implies an effect over a long period of time; this effect may result from a cumulative action of the toxicant or may result from subthreshold changes in the environment. The fact of chronic toxicity is extremely difficult to demonstrate.

The toxicity of petroleum to aquatic and terrestrial organisms is discussed in terms of its acute and chronic effects.

Acute Toxicity

The acute toxicity of oil is usually exhibited within minutes or hours of its entry into water. The acutely toxic components of oil may cause cell damage and death or interfere with the normal physiology and function of aquatic organisms. The acute effects of oil in water are generally short-lived due to rapid evaporation of the responsible components, but they can cause extensive disruption of aquatic ecosystems within a relatively short time period (225).

The immediate toxicity of petroleum hydrocarbons in water is attributed to three fractions: low boiling saturated hydrocarbons, low boiling aromatic hydrocarbons and olefinic hydrocarbons (37). Low boiling saturates with carbon chain lengths from C_1-C_8 cause anesthesia and narcosis in low concentrations and cell damage and death in high concentrations. Since short chain alkanes are more water soluble than long chain alkanes, they exhibit greater toxicity to aquatic life. The cycloalkanes are also quite toxic to aquatic organisms. Low boiling aromatics, such as benzene, toluene and xylenes, are acutely toxic to man and animals in concentrated form and in dilute solutions when they are inhaled, ingested or touched. Because aromatics are more water soluble than alkanes with an equal number of carbons, they are inherently more toxic than saturates (16). Olefins are usually present only in refined oils, especially after catalytic cracking. Olefins are more water soluble than their saturated analogs, and increase the toxicity of refined oils to aquatic organisms (225).

Some of the non-hydrocarbon components of oil are acutely toxic, as well. Cresols, xylenols, naphthols, quinolines, pyridines and carboxylic acids are highly water soluble and toxic to aquatic life (225).

Speece (316) studied 53 chemicals commonly found in petrochemical wastes for possible use as substrates for methane production and saw a definite correlation between the molecular structure of the chemicals and their toxicity to unacclimated methane-producing bacteria. Chloro substitution, aldehyde groups, double bonds, benzene rings and the position of functional groups significantly affected the toxic properties of the chemicals.

Traxler and Bernard (330) reported that high concentrations of benzene, toluene and alkyl benzenes were bacteriostatic or bacteriocidal to *Pseudomonas aeruginosa*. These compounds stimulated the growth of *Pseudomonas aeruginosa* on *n*-alkane substrates when added to the growth medium in low concentrations. These effects were apparently caused by increased permeability of the bacterial cell walls in the presence of the low boiling aromatics. Crude and refined oils were tested for their ability to inhibit ^{14}C-glucose uptake and CO_2 production in marine bacteria by Hodson, et al. (164). They reported stimulatory effects at oil concentrations up to 80 ug/l, but increasingly inhibitory effects at oil concentrations greater than 300 ug/l. The degree of toxicity of the oils was a function of their water soluble hydrocarbon content and relative enrichment with medium molecular weight hydrocarbons. Button (57) noted the lack of bacterial growth on low molecular weight hydrocarbons, except in low concentration, and attributed this effect to the disruption of functional phospholipids in bacterial membranes.

The water solubility and composition of an oil affect its initial toxicity. Walker and Colwell (352) found that utilization of ^{14}C-glucose by mixed cultures of marine bacteria was inhibited by 3 ppm of the water soluble hydrocarbons from fuel oil and 7 ppm of the water soluble hydrocarbons from crude oil. The higher concentration of naphthalene derivatives in the water soluble components of the fuel oil appeared to be responsible for the difference in the toxicity of the oils. Griffin and Calder (151) showed that the growth inhibitory effects of the water soluble components of an oil to the marine bacterium, Serratia marinorubra, were reversed in fresh medium and that no residual toxicity was carried over, indicating that the toxic effects were bacteriostatic rather than bacteriocidal. They also found that addition of yeast extract to the medium reduced the toxicity of the water soluble fractions of the oil.

Chet and Mitchell (72) discovered that kerosine interfered with the chemotactic response of bacteria to chemical attractants. Blumer (37) observed that lobsters were attracted to distillate oils and suggested that olefins and high boiling aromatic components were most likely to interfere with chemotaxis, taste reception and natural stimuli in marine organisms.

Goldacre (143) studied the effects of low boiling paraffins on the membranes of amoeba and found that the membranes expanded in the presence of low concentrations of paraffins and pulled away from the cytoplasm in the presence of high concentrations. The membrane expansion was thought to be caused by intercalation of the hydrocarbons between the lipid layers of the membrane.

Atlas and Bartha (21) reported that the lag phase of bacterial growth on fresh crude oil was extended at low temperatures. This could not be fully explained as a temperature effect, since the lag phase was shorter on weathered crude oil incubated at the same temperature. The volatile components of fresh crude oil apparently evaporated more slowly at low temperatures and exerted bacteriostatic effects over a longer period of time than at high temperatures.

Atlas (18) noted that photosynthesis and nitrogen fixation were inhibited by petroleum in water. Coffey, et al. (77) reported that the growth of freshwater algae was inhibited by water soluble extracts of fuel oil, but not by crude oil extracts. Masters and Zajic (231) observed that the growth of algae was improved in the presence of n-heptadecane, but that it was inhibited by aliphatics with chain lengths of C_{14} or less.

Busdosh and Atlas (55) studied the effects of oil on arctic amphipods and found that diesel oil was more toxic than Prudhoe Bay crude oil. The toxicity of the crude oil was associated with the paraffinic and aromatic fractions. The asphaltic fraction of the oil had no effect on the amphipods. The locomotion of amphipods and coelenterates was impaired by low concentrations of crude oil (272).

Freshwater bass lose equilibrium in water mixed with fresh crude oil, but are not affected by oil that has been stripped of low boiling, volatile components. Water soluble extracts and oil-in-water dispersions of crude and refined oils were tested for their toxicity to crustaceans and fish by Anderson, et al. (17). The water soluble extracts of crude oils contained a high percentage of aliphatics and single ring aromatics and the water soluble extracts of refined oils contained a high percentage of naphthalene derivatives. Although the total hydrocarbon concentration of the crude oil extracts was higher than that of the refined oil extracts, the refined oils were more toxic. The toxicity of the oils was a function of the concentration of di- and tri-aromatic hydrocarbons, which were 3-10 times more toxic than the benzene derivatives contained in the oils.

The above studies imply that the toxicity of oil in an aquatic environment is greatest immediately after an oil spill and that, as weathering proceeds, the solubility and, presumably, the toxicity of the oil is decreased. This may not always be the case, since chemical and biological degradation processes may increase the acute toxicity of the oil.

Larson, et al. (206) observed that yeast growth was inhibited by #2 fuel oil which had been exposed to near ultraviolet irradiation (280-400 nm). The toxicity of the oil reached a maximum after 24 hours of irradiation and appeared to be due to the formation of peroxide intermediates. Lower concentrations of phenols, carbonyls, lactones and free acids were also present in the oil after irradiation and may have contributed to the toxicity of the oil. Lacaze and deNaide (205) demonstrated that the phytotoxicity of crude oil was increased when it was exposed to artificial and natural light. The crude oil decreased the incorporation of $^{14}CO_3^{=}$ into algae by 30 per cent in the dark and by 60 per cent in the light after 3-5 days of incubation. Winters, et al. (363) found that phenalen-1-one, a compound isolated from fuel oil, inhibited algal growth and photosynthesis. The toxicity of phenalen-1-one was wavelength dependent for green algae, but was wavelength independent for blue-green bacteria.

Calder and Lader (59) showed that dihydroxynaphthalene, a microbiological degradation product of naphthalene, was more toxic to marine bacteria than the parent hydrocarbon at equivalent concentrations. The water solubility of the polar oxidation product was high enough to completely inhibit the growth of the non-oil-degrading marine bacteria, *Vibrio parahaemolyticus* and *Serratia marinorubra*. The water soluble fractions of oil had negative effects on at least one of the following bacterial growth parameters: lag time, growth rate and maximum cell density (59, 151). Possible explanations for the growth inhibitory effects of oil included blockage of substrate uptake by membranes, increased cellular leakage, formation of inhibitory compounds by co-metabolism and catabolite repression.

Atlas and Bartha (24) reported that metabolism of crude oil by two oil-degrading bacteria in pure culture ceased after two weeks for no apparent reason. They attributed the inhibitory effect to the accumulation of fatty acids in the medium. Addition of a series of fatty acids to the growth medium revealed that bacterial growth inhibition decreased as the carbon chain length of the fatty acid was increased, probably because of the decreased water solubility of long chain fatty acids. Aida and Yamaguchi (4) observed that the growth of yeasts on alkane substrates was inhibited by catabolite repression. Lauric acid was identified as the principle inhibitor of the oxidation of _n_-hexadecane to palmitic acid by yeasts. Fatty acids inhibited the growth of yeasts on alkane substrates, but not on glucose, indicating that the growth inhibition was due to catabolite repression. Dalhoff and Rehm (85, 86) showed that citric acid cycle intermediates and phosphorylated glucose inhibited the oxidation of tetradecane by _Pseudomonas_, and suggested that a non-specific catabolite repression was responsible for the inhibitory effects.

Crude oil biodegraded by an _Arthrobacter_ sp. was more toxic to sea urchins than fresh crude oil, according to Rosenberg, et al. (286). The toxicity of the degraded oil could be reduced by dialysis and was attributed to polar metabolites. Raymond, et al. (279) noted that oil spilled on soil had adverse effects on nematodes and plants after nine months. Sexstone and Atlas (294) observed an increase in polar lipoidal compounds in oil spilled on soil. These compounds were apparently not fatty acids. Zobell (382) stated that some microbial oxidation products of hydrocarbons are more toxic than the parent hydrocarbons.

Chronic Toxicity

The chronic toxicity of oil is not immediately obvious and damage to aquatic organisms may occur over an extended period of time. Some water pollutants, such as phthalic acid, have adverse effects on the reproduction of water fleas and other aquatic organisms (129). Compounds that exhibit acute toxicity in high concentration may exhibit chronic toxicity in low concentration. Aquatic or terrestrial organisms chronically exposed to oil may undergo behavioral changes or show signs of somatic or genetic damage. This section will deal with genetic damage caused by petroleum components.

Genetic toxicity and environmental mutagenesis are terms used to describe mutagenesis by chemicals that may or may not have other toxicological symptoms (311). It has been suggested that a high percentage of human cancers may be caused by mutagens in the environment (46), since a large number of known carcinogens also have mutagenic properties (10, 13). Potentially carcinogenic polynuclear aromatic hydrocarbons have been isolated from fresh crude oil (374).

According to the somatic theory of carcinogenesis, a mutagenic change in the DNA of a somatic cell is responsible for tumor production in mammals (9). If this theory is correct, crude oil in water supplies may constitute a public health threat, especially if the mutagenic and/or carcinogenic activity of the oil is increased by degradative processes. Very low concentrations of some polynuclear aromatic hydrocarbons can cause cancer in animals (38). It should be recognized that the somatic theory of carcinogenesis only predicts that carcinogens are mutagens and that not all mutagens are carcinogenic.

The carcinogenic activity of petroleum hydrocarbons has been studied extensively. Wallcave, et al. (356) compared the tumorigenic activity of petroleum asphalts and coal tar pitches and found that the coal tar pitches induced skin tumors in over 90 per cent of 218 mice, while petroleum asphalts induced skin tumors in only 3 per cent of an equal number of mice. Coomes (80) reported that there was little difference in the carcinogenic potential of shale oils and crude petroleum. The high boiling components of petroleum have the greatest carcinogenic potential. Wilbur (360) stated that all crude oils or products containing hydrocarbons boiling between 300-500 C should be regarded as potentially carcinogenic substances. Only six of 234 hydrocarbons positively identified in a reference oil by Mair (222) have been listed as suspected carcinogens (335, 336).

Berenblum and Schoental (33) fractionated coal tar on an alumina column with various solvents. Light petroleum eluates from the column were not carcinogenic to mice, but benzene eluates were carcinogenic. Vacuum distillation of the coal tar showed that the distillate fractions containing benzo(a)pyrene and the fractions distilling immediately before and after benzo(a)pyrene induced tumors on mouse and rabbit skin. Synthetic crude oil derived from coal tar was fractionated into 14 acidic, basic and neutral fractions and tested for mutagenic activity by the Ames test (288). Frameshift mutagens were detected in the water soluble, strong acid fraction, the ether soluble base fraction and the hexane, hexane-benzene and benzene-ether fractions. Bingham, et al. (35) found carcinogenic activity in the naphthenic fraction of cutting oil, but not in the paraffinic fraction. A comparison of the 4- and 5-ring polynuclear aromatic hydrocarbons in the cutting oils showed that 4-ring compounds had the greatest carcinogenic activity. Cook, et al. (79) recovered the majority of the carcinogenic activity from three crude oils in the 350-400 C boiling range. They recovered highly carcinogenic tetracyclic thiophene derivatives from the oils by solvent fractionation, but found little carcinogenic activity in the solvent insoluble fraction. A cracked petroleum residue was fractionally distilled and the fractions were tested for carcinogenic activity in mice by Tye, et al. (333), as shown in Table 6.

Wynder and Hoffman (371) reported that as little as one microgram of the carcinogenic polynuclear aromatic hydrocarbons from cigarette smoke condensates could transform normal mammalian

Table 6. Boiling point distribution and potency of carcinogens in a cracked petroleum residuum (333).

Fraction	%	Endpoint (°C)	Characteristic constituents	Carcinogenic* potency
Initial residuum	100.0	-	-	0.45
1, 2, 3	30.3	349	Trace 3-ring Benzenes Naphthalenes	0.00
4	9.5	363	3-ring Trace pyrene	0.06
5	9.8	388	15% phenanthrene 1-2% pyrenes	0.36
6	9.6	404	Phenanthrenes Benzfluorenes Pyrenes	0.52
7	9.9	426	4-ring	0.80
8	9.4	438	Alkyl 4-ring	0.68
9	2.3	455	4, 5-ring	0.80
10	19.2	455	5-ring, larger	0.44

*Carcinogenic potency of each fraction expressed as fractional number of tumors produced on 20 male CH3 mice after repeated skin application relative to that produced by 1.0 mg/week of 3-methylcholanthrene in 29 weeks.

cells into tumor cells. The benzo(a)pyrene content of the condensates was positively correlated with their tumorigenic activity. The alkylated 4- and 5-ring components in cigarette smoke condensates were potent inducers of tumors in animals also.

Methylated derivatives of polynuclear aromatics are usually more carcinogenic than their unmethylated counterparts (159, 250, 360, 371). The temperature at which polynuclear aromatic hydrocarbons are formed largely determines their degree of alkylation. Polynuclear aromatics formed at low temperatures, such as those in coal and petroleum, are highly alkylated, while high temperature combustion products yield fewer alkylated derivatives (38). Heidelberger (159) listed a series of carcinogenic polynuclear aromatic hydrocarbons in decreasing order of potency as follows: 20-methylcholanthrene, 9, 10-dimethyl-1, 2-5, 6-dibenzanthracene, 9, 10-dimethyl-1, 2-benzanthracene, 3, 4-benzpyrene, 1, 2-5, 6-dibenzanthracene, 10-methyl-1, 2-benzanthracene, 4-fluoro-10-methylbenzanthracene and 4-methyl-1, 2-benzanthracene. This list shows the effect of methylation on the potency of known carcinogens.

Polynuclear aromatic hydrocarbons are tumor inducers and require the presence of tumor promoters to act as carcinogens. Volatile phenols, aldehydes and acids may serve as tumor promoters for polynuclear aromatics (371). Carruthers, et al. (62) isolated 1, 2-benzanthracene derivatives from Kuwait mineral oil, but found only marginal carcinogenic activity in these compounds in the absence of promoters. Elemental sulfur and organic sulfur compounds in oil were found to act as accelerators of tumor production by polynuclear aromatics in animals by Horton, et al. (170, 171). Less than half of the carcinogenic potency of oil was caused by the tumor inducers without promoters. Some long chain hydrocarbons and polar compounds, such as ketones and NH-carbazoles, were thought to act as tumor accelerators as well.

Carcinogenic polynuclear aromatics are widely distributed in aquatic environments. Endogenous synthesis by plants and animals, industrial effluents and atmospheric precipitates all contribute to the environmental load of these compounds. Bacteria can both synthesize and degrade polynuclear aromatics (202, 385). Marine organisms are capable of concentrating carcinogenic hydrocarbons, but there is no clear evidence that this constitutes an environmental cancer hazard (319). Borneff and Fisher (40) isolated and identified 3, 4-benzpyrene and several other potentially carcinogenic hydrocarbons from rapid filters in a water treatment plant. They considered that the level of carcinogens in treated water was too low to present a health hazard, but warned that increased pollution may increase the concentration of carcinogens in water to hazardous levels. Zobell (384) reported wide variations in the benzo(a)pyrene content of natural substances, such as soil, water, food, etc. The U. S.

Environmental Protection Agency recovered several known carcinogens, including polynuclear aromatic hydrocarbons, from drinking water concentrates (338). Domestic wastewater may carry from 1-100 ug/l of polynuclear aromatic hydrocarbons, 10-40 per cent of which are carcinogenic (321).

Crude oil hydrocarbons are relatively inert, and generally must be enzyme activated before they become mutagenic or carcinogenic. Benzene is known to cause leukemia when chronically inhaled (343). The benzene molecule is probably not the direct cause of leukemia, since oxidation products of benzene, such as phenol and pyrocatechol, are more toxic to white blood cells. Mechanisms proposed to explain the leukemogenic activity of benzene include somatic cell mutations, latent virus activation and immune impairment.

Ames (14) determined that epoxides of polynuclear aromatic hydrocarbons were mutagenic to bacteria, but found little mutagenic activity in the parent hydrocarbons and dihydrodiol intermediates. The epoxide derivatives intercalated between DNA base pairs and reacted covalently with the DNA. Maher, et al. (221) showed that carcinogenic polynuclear aromatics reacted covalently with transforming DNA isolated from *Bacillus subtilis*, reducing its transforming activity and increasing the forward mutation rates in transformed bacteria.

Activation of polynuclear aromatic hydrocarbons to carcinogens by mammalian enzymes has been well documented. Daly, et al. (87) detected increased monooxygenase and epoxide hydrase activity in the microsomal fraction of mouse liver induced with 3-methylcholanthrene. The level of cytochrome P-450 was increased in the soluble fraction of the mouse liver. The epoxide hydrase detoxifies epoxide derivatives of polynuclear aromatic hydrocarbons by converting them to dihydrodiols. Cytochrome P-450 acts in conjunction with monooxygenases to form epoxide intermediates from the parent hydrocarbons. Epoxide hydrases have been found in purified cytochrome P-450 and P-448 extracts, indicating that a coupled monooxygenase-epoxide hydrase system may exist to detoxify epoxides as they are formed. In addition to being enzymatically converted to dihydrodiols, epoxides may be non-enzymatically converted to phenols or form conjugates with glutathione and the purine bases of nucleic acid.

The mutagenic, carcinogenic and cytotoxic effects of several aromatic and olefinic compounds appear to be caused by the reaction of their epoxide intermediates with tissue constituents. Epoxides of K-region polynuclear aromatic hydrocarbons are especially reactive. The electron density of the K-region is thought to influence its reactivity in biological systems. Figure 1 shows the major differences in the early degradation steps of a typical K-region polynuclear aromatic hydrocarbon by mammals and microorganisms (139, 141).

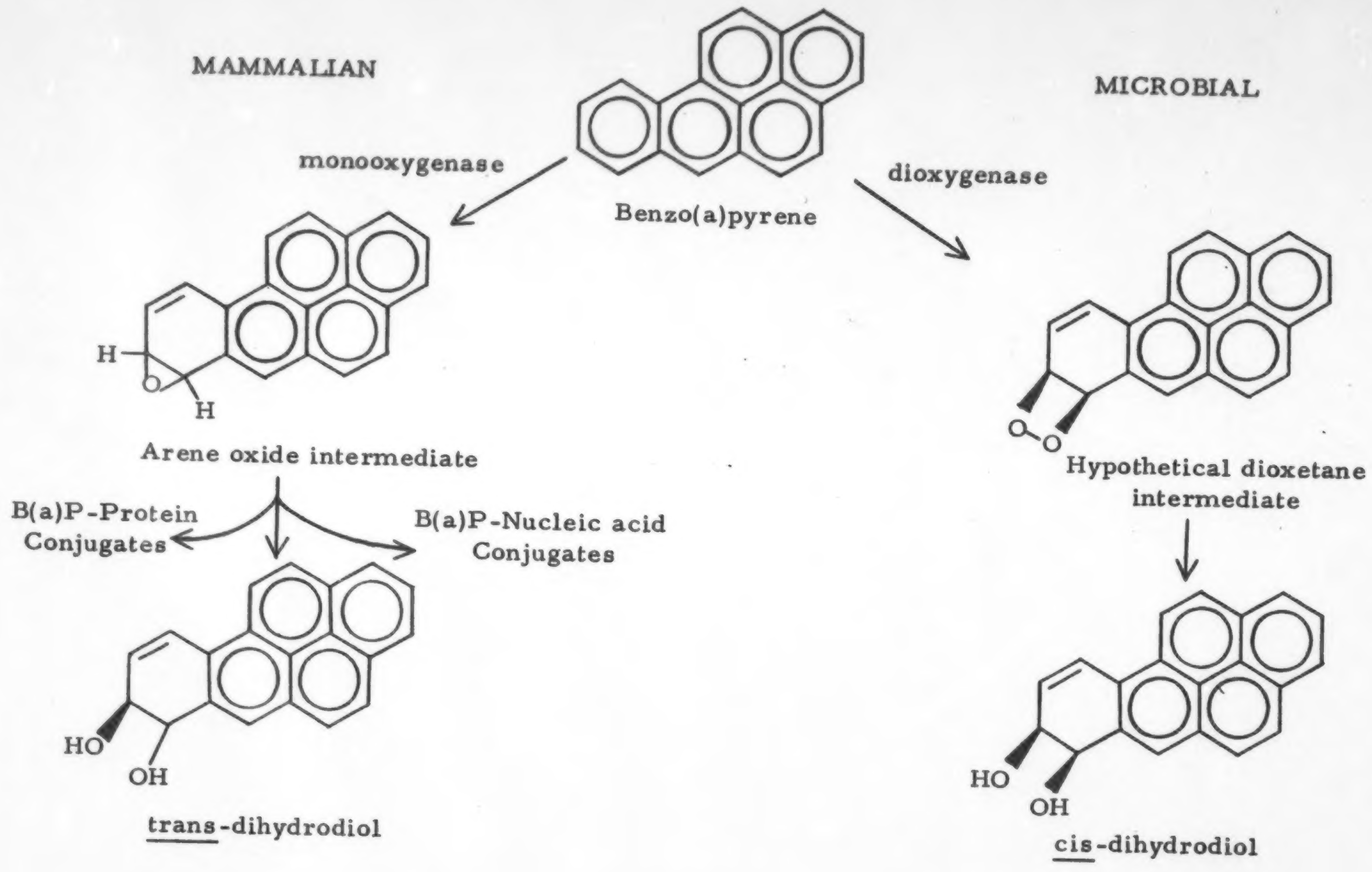
MAMMALIAN
MICROBIAL
monooxygenase
Benzo(a)pyrene
dioxygenase
H
O
H
Arene oxide intermediate
B(a)P-Protein Conjugates
B(a)P-Nucleic acid Conjugates
HO
OH
trans-dihydrodiol
O-O
Hypothetical dioxetane intermediate
HO
OH
cis-dihydrodiol

Figure 1. Comparison of mammalian and microbial degradation of benzo(a)pyrene.

Although there are similarities in the degradation of polynuclear aromatics by mammals and microorganisms, the microbial intermediates apparently do not have increased carcinogenic activity, in contrast to the mammalian intermediates. The production of cytochrome *P*-450$_{cam}$ by *Pseudomonas putida* during the degradation of camphor is analogous to the formation of cytochrome *P*-450$_{LM}$ in rabbit liver after induction with phenobarbital. The amino acid compositions of these two cytochromes were compared by Dus, et al. (108) and found to be quite similar. Immunological cross reaction occurred between the two cytochromes, but they differed in catalytic and physical properties. The closest similarity between mammalian and microbial enzyme systems was reported by Ferris, et al. (122), who discovered monooxygenase activity in the fungus, *Cunninghamella baineri*, which yielded products from aromatic hydrocarbons similar to those of mammalian monooxygenases. Epoxide and *trans*-dihydrodiol intermediates were produced from aromatic hydrocarbon substrates and evidence of epoxide hydrase activity was detected. Cytochrome *P*-450 was not detected, but benzpyrene hydroxylase activity in sonicated enzyme extracts was inhibited by carbon monoxide, indicating the presence of a CO-inhibited cytochrome with properties similar to that of cytochrome *P*-450.

Several reports have stated that bacteria, plants and animals are capable of synthesizing carcinogenic polynuclear aromatic hydrocarbons, such as benzo(a)pyrene (202, 354), but the majority of reports indicate that the carcinogenic activity of polynuclear aromatics is destroyed by microorganisms (93, 276, 305). Microbial oxidation of polynuclear aromatics is relatively slow, but mixed cultures or co-oxidation reactions may alter their chemical structure enough to render them non-toxic, since only minor changes can affect the carcinogenic potency of these compounds (384).

Polynuclear aromatic hydrocarbons are the most widely recognized carcinogens in petroleum, but they are not the only mutagenic and/or carcinogenic compounds in oil, especially after chemical or biological degradation of the oil has occurred. Peroxides are characteristic products of hydrocarbon autooxidation (357) and may be involved in tumor production in mammals (295). Larson (206) recovered peroxides, carbonyls, phenols, lactones and free acids in fuel oil after it had been photooxidized by ultraviolet light. Certain lactones and peroxides are known to have carcinogenic activity (340). DeMik and DeGroot (95) induced chromosome breaks in Escherichia coli with ozonized cyclohexene and proposed that a peroxide intermediate was responsible. Scholes, et al. (292) reported that hydroperoxide derivatives of nucleic acids were formed after irradiation with X-rays and ultraviolet light. The hydroperoxides were associated with pyrimidine bases only. Updegraff and Bovey (334) showed that organic hydroperoxides were reduced by microorganisms and animal tissues by hydrogenylase enzymes and antioxidants, respectively. Peroxides may aid in the covalent linkage of polynuclear aromatic hydrocarbons and DNA (295).

Alkylating agents, such as nitrogen and sulfur mustards, epoxides, ethyleneimines, methyl sulphonates and certain lactones may exhibit both mutagenic and carcinogenic properties. Mono- and bi-functional alkylating agents cause base-pair substitutions and deletions in DNA by reacting with the nitrogen atoms in the bases (285). Bi-functional alkylating agents are more active as mutagens, as they can cross-link between DNA strands and cause large deletions. Mono-functional alkylating agents are generally strong mutagens but weak carcinogens, causing point mutations in single DNA strands (48). Linear diepoxides with one to four carbons between epoxides have carcinogenic and mutagenic activity. Epoxides separated by 4.4-9.0 Å are able to cross-link DNA strands, while epoxides separated by 11-12 Å cannot cross-link DNA. Malonaldehyde, a major product of polyunsaturated lipid peroxidation in animals, is mutagenic to bacteria (253, 295). Malonaldehyde cross-links between guanosine and cytosine base pairs, causing frameshift mutations in a manner similar to the mutagenic antibiotic, mitomycin C. The CoA derivative of malonaldehyde, malonyl semialdehyde CoA, is formed anaerobically by Clostridium kluyveri during the fermentation of pyruvate to succinate (103).

Certain types of lactones have mutagenic and carcinogenic activity (295). Beta-lactones, containing a strained, four-membered heterocyclic ring, are more reactive than alpha- or gamma-lactones, and may undergo hydrolysis, alcoholysis, aminolysis and polymerization reactions (380). Some five- and six-membered ring lactones also have carcinogenic activity. The activity of the carcinogenic antibiotics, patulin and penicillic acid, is attributed to the reaction of their lactone rings with the sulfhydryl groups of proteins (98, 99, 136). Carcinogenic lactones may also react with guanosine in DNA (98). Strained, three-membered heterocyclic rings, such as epoxides and ethyleneimines, react with proteins and nucleic acids in a manner similar to lactones. The lactone ring is broken during reaction with sulfhydryl groups or nucleic acid base-pairs, and must be intact for these compounds to be mutagenic or carcinogenic.

Aflatoxin B_1, a potent carcinogen and mutagen, is inactivated by bacteria and fungi which are able to cleave its lactone ring (74, 190, 193). The mutagenic antibiotic, actinomycin D, is also inactivated by lactone ring cleavage (193, 210). Lactone intermediates are formed in the ortho ring cleavage pathway of catechol degradation, but it is not likely that they are mutagenic, as they are common microbiological intermediates of aromatic hydrocarbon degradation (103). Mutagenic lactones may enter water in industrial waste effluents or may be formed by photooxidation of oil (206).

The strained rings of lactones, epoxides, etc. react preferentially with the sulfhydryl groups of proteins rather than nucleic acids. It is not clear whether the carcinogenic activity of these compounds in animals is caused by their binding to proteins or nucleic acids. Heidelberger (159) found that the epoxides of carcinogenic polynuclear aromatic hydrocarbons reacted with a specific soluble protein in mouse skin and found no evidence of their reaction with nucleic acids. Others have reported the binding of epoxides directly to DNA (14,221).

Aromatic nitroso compounds, nitrosamines and other nitrogen-containing compounds have been found to be mutagenic to bacteria by Ames, et al. (13). Kier, et al. (192) tested cigarette smoke condensates for mutagenic activity by the Ames test and found that condensates of air-cured tobacco were mutagenic only after liver microsome activation, but that condensates of nitrate-cured tobacco were mutagenic without enzyme activation. N-nitroso compounds are enzyme activated to mutagens and carcinogens, possibly by conversion to o-aminophenol derivatives (213,214,220). An elevated level of o-aminophenols in the urine of men and animals with abnormal tryptophan metabolism caused by bilharziasis may be responsible for their increased incidence of bladder cancer (2, 50). The o-aminophenol derivatives of nitrosamines do not react with sulfhydryl groups until they are oxidized to o-quinoneimines, indicating that several steps are involved in the activation of nitrosamines to carcinogens (75,220).

Carcinogens containing nitro groups generally require enzyme activation to exhibit mutagenic activity. Both mammalian and bacterial nitro reductases are capable of activating nitro carcinogens, such as nitrofurazone and furylfuramide, directly to mutagens (13). Bacterial nitro reductases are most active under anaerobic conditions. McCoy, et al. (239) demonstrated that cell-free extracts of anaerobic bacteria activated the procarcinogen, 2-aminofluorene, to a mutagen. Nitrosamines are inactive in the Ames test unless they are converted to the nitrosamide form. Activation of dimethylnitrosamine is accomplished by liver microsomes, but not by bacterial enzymes (13).

Nitrosamines may be formed in several ways, including *in vivo* and *in vitro* production by *Proteus mirabilis* and other microorganisms (47), reaction of secondary amines with nitrites in meat during cooking or in the stomach under acid conditions (213). It has been suggested that the incidence of gastrointestinal cancer may be increased by the ingestion of foods containing nitrates and nitrites. Once nitrosamines are formed, they are extremely stable and resistant to microbial degradation (324).

Chloramines can cause chromosome breaks in bacterial DNA (207,299). These compounds are formed by the reaction of amines with chlorine in chlorinated wastewater (338). Microbial degradation of hydrocarbons in the presence of excess nitrogen results in the formation of amines. The production of nitrosamines and chloramines from petroleum may require a combination of biological and non-biological reactions.

Hydrocarbon carcinogenesis was first recognized in 1775 among chimney sweeps who had a high incidence of scrotal cancer. The aromatic hydrocarbons were first implicated as carcinogens in soot and the role of aliphatic hydrocarbons in the production of skin cancer was not discovered until later among cotton spinners exposed to spindle oil. The value of washing with soap and water for reducing the incidence of skin cancers after exposure to hydrocarbons was noted among chimney sweeps with good hygienic practices (243).

Hueper (174) studied the site of action of a wide variety of carcinogens in mammals and found that they were specific for certain organs. Aliphatic and aromatic epoxides are suspected of causing cancer of the skin, lung and blood-forming tissues. The suspected targets of chlorinated hydrocarbons are the skin, lung and liver. Nitrosamines may affect the lung, liver, kidney, bladder and esophagus. Paraffins, waxes, asphalts and mineral oils from petroleum are known to cause skin and scrotal cancers and are suspected of causing lung and larynx cancers. Aromatic amines, such as naphthylamines, cause cancer of the bladder, ureter, renal pelvix and possibly the lung, liver and prostate.

Hueper (175) found that repeated exposure to low levels of carcinogens was a more effective means of tumor induction in mammals than large single doses. Numerous experiments have confirmed the danger of continuously ingesting low concentrations of carcinogenic polynuclear aromatic hydrocarbons in food and water. Repeated doses of carcinogens are thought to overwhelm the natural detoxifying mechanisms of the host.

Hueper and Ruchhoft (176) showed that carbon adsorbates of raw river water polluted with oil refinery effluents were weakly carcinogenic to mice. Russell and Kotin (289) observed an increased incidence of squamous papillomas about the mouth of white croakers obtained from the ocean within two miles of a sewage outfall, but saw no tumors in white croakers 50 miles from the outfall. Since the white croaker is a bottom feeder, mouth abrasions were thought to be a contributing factor to tumor production in the fish in polluted water. Payne, et al. (270) showed that used crankcase oils were mutagenic to bacteria in the Ames test after liver microsome activation. Mutagenic activity was detected in Mississippi River water in areas exposed to pollution from chemical and oil refineries by Pelon, et al. (271). Page, et al. (267) reported an increased incidence of gastrointestinal cancers in Louisiana parishes obtaining their drinking water from the lower Mississippi River compared to those parishes using well water. It is evident that water highly polluted with industrial effluents poses an increased public health threat. The amount of mutagenic and/or carcinogenic substances in water contributed by petroleum products is not known and the role of chemical and biological processes in enhancing the toxicity of oil in water is not clear.

Mutagenicity Screening Tests

Mutagenicity tests were originally developed to detect chemical mutagens, but the discovery that a high percentage of carcinogens are mutagenic in a wide variety of mutagenicity tests has led to their use as an aid in the selection of potentially carcinogenic chemicals for priority animal testing (13, 46). The short-term screening tests are relatively simple, rapid and inexpensive to perform in comparison to animal tests and some appear to be able to predict the carcinogenic potential of chemicals with a high degree of accuracy. Since not all mutagens are carcinogenic, the short-term screening tests for potential carcinogens are not definitive and are not designed to replace animal testing for carcinogens.

The short-term mutagenicity screening tests depend on observable changes in the phenotype, chromosomal material or morphology of cells for the detection of mutagens. Both procaryotic and eucaryotic test systems, such as bacteriophage, bacteria, yeasts, fungi, fruit flies and tissue cultures, have been employed to detect

Table 7. Short-term mutagenicity screening tests for carcinogens.

Test system	Scoring	References
Viruses		
T4r	Plaque morphology mutations	104
Lambda	Prophage induction in *E. coli* K-12	160, 329
Transforming DNA		
Bacillus subtilis	Chromosome breakage	299
	Loss of transforming activity	221
	Tryptophane synthetase mutation	373
Bacteria		
Bacillus subtilis (rec$^-$)	Lethality - recombinant DNA deficient	186
Salmonella typhimurium (his$^-$)	Reversion to histidine prototrophy	7, 13
Escherichia coli (pol A$^-$)	Lethality - lacks DNA polymerase I	306
Escherichia coli (trp$^-$)	Reversion to tryptophan prototrophy	149
Escherichia coli B/Sd	Reversion to streptomycin dependence	94, 322
Escherichia coli B/r	Forward mutation to T1 phage resist.	94, 364
Yeasts		
Saccharomyces cerevisiae	Adenine deficient, red pigment, mitotic segregation	251, 381
Schizomyces pombe	Adenine deficient, red pigment	251, 381

mutagens (125). Table 7 lists some of the mutagenicity tests that have been developed.

Plaque morphology changes and prophage induction have been used as the basis of bacteriophage screening tests for mutagens. Ames, et al. (14) found that epoxides of K-region polynuclear aromatic hydrocarbons caused changes in the plaque morphology of T2 bacteriophage. Thompson and Woods (329) induced lambda prophage in a lysogenic strain of Escherichia coli K-12 with carcinogenic N-nitrosamines.

Bacterial screening tests utilize forward and back mutations to detect mutagens. Witkin (364) described a mutagenicity test in which the resistance of E. coli B/r to T1 bacteriophage was increased in the presence of mutagens. Szybalski (322) and Demerec (94) utilized a screening test in which streptomycin-dependent strains of E. coli were back mutated to streptomycin-independence when exposed to mutagens. Ames (7) developed a series of Salmonella typhimurium mutants which are reverted from histidine auxotrophy to protrophy by chemical mutagens. Maher, et al. (221) observed decreased transformation rates and increased forward mutation rates in the transforming DNA of Bacillus subtilis after exposure to carcinogenic polynuclear aromatic hydrocarbons. Back mutation tests are generally preferred over forward mutation tests for mutagen detection, because they are faster and easier to perform and have

Table 7. (Continued)

Test system	Scoring	References
Fungi		
Neurospora crassa	Adenine deficient, purple pigment Recessive lethal mutations	96
Aspergillus nidulans	Chromosome non-disjunction, crossing-over	34
Invertebrates		
Drosophila melanogaster	Phenotypic mutations, sex-linked lethals in fruit flies	26, 310
Bombyx mori	Mutations in silkworm oocytes	326
Habrobracon	Dominant lethals in parasitic wasp	308
Plants		
Vicia	Chromosome aberrations in horse bean root tips	194
Allium	Chromosome aberrations in onion root tips	194
Hordeum vulgare	Chromosome aberrations in barley	370
Vertebrates		
Mice	Specific locus phenotypic mutations	63
Rats and mice	Dominant lethal mutations	27
Mice	Sebaceous gland suppression	39

Table 7. (Continued)

Test system	Scoring	References
Mammalian cells		
Mouse bone marrow cells	Micronucleus test - chromosome aberrations	291
Mouse sperm cells	Chromosome and morphological aberrations	372
Mouse lymphocytes	Chromosome aberrations	31
Guinea pig fetal cells	Fibroblast transformation	113
Rat liver cells	Epithelial cell transformation	247
Microsomal membranes	Degranulation of rough endoplasmic reticulum	362
Mouse lymphoma cells	Specific locus mutation - thymidine kinase gene	76
Chinese hampster ovary cells	Specific locus mutation - hypoxanthine-guanine phosphoribosyl transferase gene	73, 76
Cultured human cells	Unscheduled DNA synthesis - ^{3}HTdR uptake	290, 318
Rat lymphocytes	Sister chromatid exchange - Chromosome aberrations	215, 273
Cultured mammalian cells	Plasminogen activator test for tumor promoter activity	131

Table 7. (Continued)

Test system	Scoring	References
Host-mediated assay		
Microbial cells	Mutagen activation in mouse	209
Mammalian cells	Mutagen activation in mouse	45, 76

increased sensitivity to mutagens due to lower spontaneous mutation rates.

Shirasu, et al. (301) compared several microbial mutagenicity tests and found that a Rec$^-$ strain of *Bacillus subtilis*, deficient in recombinant DNA repair, was the most sensitive test system for detecting mutagenic pesticides. Mutagens are detected in the "rec-assay" by comparing the lethality of the chemicals to Rec$^+$ and Rec$^-$ strains of *Bacillus subtilis*. Chemicals which are more lethal to Rec$^-$ than Rec$^+$ strains, as measured by zones of inhibition, are considered mutagenic in this test. *E. coli* WP2 histidine auxotrophs and *Salmonella typhimurium* LT2 histidine auxotrophs detected only 9 of 166 pesticides as mutagens by back mutation compared to 23 detected by the Rec$^-$ test. A Pol$^-$ strain of *E. coli*, deficient in DNA polymerase I. is nearly as sensitive as the Rec$^-$ strain of *B. subtilis*. The principle of the Pol$^-$ test is the same as the Rec$^-$ test, in that both tests detect mutagens by their lethality (46).

The yeast mitotic recombination test detects gene shuffling induced by mutagens during chromosome replication which causes an adenine deficiency in yeast. The adenine deficient cells form a red pigment, while the normal cells are not pigmented. An increase in the number of red yeast colonies after exposure to a chemical indicates that it is mutagenic. This test is relatively insensitive to most mutagens, possibly because the mutagens do not readily penetrate the yeast cell walls (125, 131).

Auerbach (26) was the first to successfully demonstrate chemical mutagenesis in fruit flies with mustard gas in 1941. The fruit fly, *Drosophila melanogaster*, is used in a live animal test for mutagen detection and has an advantage over most tests, since it allows *in vivo* enzyme activation of the chemicals tested. The bacterial test systems generally employ mammalian liver microsomes to activate promutagens to mutagens. Enzyme activation by fruit flies is similar to that of mammals (131). Mutation of a specific sex-linked recessive lethal gene in fruit flies is the basis of a recently developed mutagenicity screening test (310).

Unscheduled DNA synthesis is a promising technique for detecting mutagenic damage in cultured mammalian cells because of its high sensitivity. DNA with mutagen-induced breaks is allowed to incorporate radioactive DNA precursors into its strands by repair replication and the radioactivity of the DNA is measured to determine the amount of genetic damage caused by a chemical mutagen (131).

Cultured mammalian cells are used to detect mutagens by a variety of other means. Mouse lymphoma cells and Chinese hamster ovary cells are used to detect mutations that increase the resistance of the cells to specific DNA inhibitors. Sister chromatid exchange is a method of detecting mutagen-induced chromosome breaks in mammalian cells. In this test, Chinese hamster ovary cells are treated with bromodeoxyuridine during replication, which decreases the affinity of their chromosomes to dye. Chromosome breakage by mutagens increases the amount of recombinant DNA repair in the damaged chromosomes. The repaired areas of the chromosomes take up more dye and an increase in the number of dye patches in the chromosomes indicates mutagen damage. Mammalian cell transformation tests are a more direct means of testing the carcinogenic potential of chemicals. Mouse lymphoma and Chinese hamster ovary cells have been used in transformation tests. The number of cell foci in a monolayer of cultured cells is counted as a measure of mutagenic activity (131).

Cell culture techniques have also been used to detect the tumor promoting activity of chemicals which increase the carcinogenicity of other chemicals but are not carcinogenic by themselves. The plasminogen activator test detects the presence of plasminogen, a protein that degrades fibrin clots in blood, in transformed mammalian cells (131).

No single test system is adequate to detect the mutagenic activity of a wide range of chemicals. The use of batteries of short-term mutagenicity screening tests has been proposed to confirm the mutagenicity of chemicals. A major problem with this approach is that the test batteries do not always agree. The sensitivity of various test systems is variable, so that strong mutagens generally give good agreement among test systems, but weak mutagens do not agree as well. For example, a battery of 12 short-term mutagenicity screening tests was used to determine the mutagenic potential of the suspected carcinogen, saccharin (131). Saccharin was not mutagenic in nine of the tests and was weakly mutagenic in three: two sister chromatid exchange tests and a mouse lymphoma cell mutation causing increased resistance to DNA inhibitors. None of the microbial cell tests were positive. The National Academy of Sciences (259) found that 5 of 21 screening tests, including a plasminogen activator test, indicated that saccharin was mutagenic.

A comparison study of mutagenicity screening tests (46) showed that the carcinogenicity and non-carcinogenicity of 120 chemicals was accurately predicted for 90 per cent of the chemicals by bacterial mutation tests using liver microsome activation, 83 per cent by cell transformation tests, 72 per cent by endoplasmic degranulation tests, 70 per cent by cellular morphology changes following subcutaneous implantation, 90 per cent for polynuclear aromatic hydrocarbons, but only 52-62 per cent for other chemicals by the sebaceous gland suppression test and 62 per cent by the tetrazolium reduction test in mouse skin.

Description of the Ames Test

One of the most successful short-term screening tests is the *Salmonella* microsome/mutagenicity test developed by Ames (13) as a by-product of his work on the histidine operon of *Salmonella typhimurium* (158). The test has been in development since 1964 (131) and improvements to the test have been made in the process (5, 7, 8, 12, 13, 238, 264).

All of the tester strains used for mutagenicity testing in the Ames test are derived from *Salmonella typhimurium* LT2 parent stock. Each mutant strain has a specific histidine mutation which is back mutated from histidine auxotrophy to histidine prototrophy in the presence of mutagens. Some of the strains are reverted to histidine protrophy by base-pair substitution mutagens and others by frameshift mutagens, making the test specific for the type of mutagens it detects. Other mutations have been introduced into the strains to increase their sensitivity to low levels of mutagens (Appendix 4).

Several improvements in the tester strains have resulted in a mutagenicity test that has a high degree of sensitivity and specificity for a wide range of mutagens. Five standard tester strains are currently recommended for general mutagenicity testing: TA 100, TA 1535, TA 1537, TA 1538 and TA 98. The sensitivity of these tester strains to low levels of mutagens has been increased in six ways as described below:

1. The histidine mutations occur at mutational "hot spots" within the histidine genes. The genetic regulation of histidine synthesis is controlled by nine genes, some of which are highly susceptible to mutation (131, 158). The existence of mutational hot spots has also been reported in other microorganisms. Okada, et al. (265) observed an unusually high reversion frequency in T4 bacteriophage containing a frameshift mutation in the lysosome gene. The hot spot was tentatively identified as a sequence of six adenine bases. Dickson, et al. (100) described sequence blocks in the lac operon of bacteria with higher numbers of G-C and A-T base pairs than normal and considered them to be possible sites of frameshift mutations.

One of three different mutations causing histidine auxotrophy is contained in the five standard tester strains. These mutations determine the sensitivity of the strains to mutagens and their specificity for base-pair substitution or frameshift mutagens. Salmonella tester strains TA 100 and TA 1535 contain the hisG46 missence mutation which is reverted by base-pair substitution mutagens, but not by frameshift mutagens. Tester strain TA 1537 contains the frameshift mutation, hisC3076, which has a $\begin{matrix}\mathrm{GGGG}\\\mathrm{CCCC}\end{matrix}$ sequence that is reverted to histidine prototrophy by a -1 deletion of a $\begin{matrix}\mathrm{G}\\\mathrm{C}\end{matrix}$ base pair by frameshift mutagens. Deletions of this type are induced by epoxides of carcinogenic polynuclear aromatic hydrocarbons. Tester strains TA 1538 and TA 98 contain the frameshift mutation, hisD3052, which has a repetitive $\begin{matrix}\text{CGCGCG}\\ \text{GCGCGC}\end{matrix}$ sequence that is reverted to histidine prototrophy by a -2 deletion of base pairs. Enzyme activated aromatic nitroso derivatives, benzo(a)pyrene, aflatoxin B_1, etc., are capable of reverting these strains to histidine prototrophy (12).

2. Deletion of the galactose gene in the *Salmonella* tester strains has increased their permeability to large molecules. This deletion results in a deep rough (rfaE) mutation in the lipopolysaccharide (LPS) of the tester strains (12). The intact LPS layer of the bacteria normally acts as a barrier to the penetration of large molecules into Gram-negative bacteria (212). The natural resistance of Gram-negative bacteria to the harmful effects of many chemicals is due to the semi-permeable nature of the lipopolysaccharide, phospholipid and protein layers in their cell walls (110). The cell walls of *Escherichia coli* are impermeable to most chemicals for which there is no specific transport mechanism (232).

Several factors are known to affect the permeability of bacterial cell walls. Egan and Russell (110) described *E. coli* K-12 conditional mutants that were temperature sensitive at 42 C, but could grow at the restrictive temperature if the osmotic pressure of the medium was increased with sodium chloride or sucrose. The strains exhibiting this characteristic were called temperature sensitive, osmotic remedial (TSOR) mutants. These mutants had increased permeability to sodium desoxycholate, methylene blue, acridine orange, triphenyl tetrazolium chloride, actinomycin D, novobiocin, rifampicin, ampicillin and other growth inhibitory chemicals at the restrictive temperature, but not at non-restrictive temperatures. Other factors which affect the permeability of bacteria include infection with a derepressed sex factor, unbalanced growth due to a defect in protein synthesis, loss of cell wall components by chemical treatment, freezing and drying (110, 232).

Gram-negative bacteria with intact LPS layers may exhibit permeability changes due to other factors. A series of tol mutations regulate the tolerance of bacteria to colicins and bacteriocins (110). Normark and Westling (261) and Nakamura (257) reported that an envelope mutant of *E. coli* (envA) with an intact LPS layer had increased permeability to basic dyes, such as crystal violet. They also found that inhibition of nucleic acid synthesis in wild type *E. coli* with nalidixic acid decreased dye uptake and inhibition of protein synthesis by chloramphenicol increased dye uptake. A coordinate regulation of nucleic acid synthesis and permeability was suggested to explain these effects (232).

Wilkinson, et al. (361) described a series of *Salmonella* typhimurium LT2 rough (rfa) mutants with LPS deficiencies. The deep rough (rfaE) mutants employed by Ames in the *Salmonella* mutagenicity test are unable to add heptose sugars to the keto-deoxyoctanoate (KDO) of the core LPS, resulting in the loss of all proximal sugar units in the LPS. This is presumably the largest LPS deletion which is not lethal to Salmonella typhimurium (12). Loss of the entire O-antigen and part of the core LPS makes the mutant cells more permeable to bile salts, crystal violet, antibiotics, and other large ring structures. The sensitivity of the Salmonella tester strains to mutagens with large ring structures, such as polynuclear aromatics with four or more rings, is greatly increased by the deep rough LPS mutation. A comparison of the sensitivity of Salmonella tester strains with normal LPS, partial LPS and deep rough LPS mutations to mutagens showed that as the LPS layer was decreased the number of histidine revertants increased (12, 13). Another advantage of the rfaE mutation in the Salmonella tester strains is that removal of the O-antigen renders the strains non-pathogenic.

3. A mutation in the uvrB gene of the Salmonella tester strains results in the loss of a DNA excision repair mechanism and increases the number of histidine revertants in the presence of mutagens. Witkin (365) stated that nearly all DNA damage caused by chemicals or short wavelength irradiation is repaired by the excision and recombination repair mechanisms in bacteria, resulting in low frequencies of mutation. Maher, et al. (221) suggested that a possible relationship exists between the systems responsible for repair of ultraviolet light damage and hydrocarbon cross-linkage damage in DNA. Ultraviolet light causes cross-linkage of DNA

strands by formation of thymidine dimers and may induce mutations in bacteria in a manner similar to chemical cross-linking agents. Salmonella tester strains lacking uvrB excision repair are 10-100 times more sensitive to alkylating agents than strains with normal repair mechanisms (7).

4. Salmonella tester strains with elevated levels of recombination repair enzymes have been selected to increase the sensitivity of the mutagenicity test (131). McCann, et al. (238) reported that mutagens are more effective in the presence of an error-prone recombination repair system. In trying to repair DNA damage, the recombination repair system fixes the back mutation into the tester strains and increases the number of histidine revertants. Witkin (365) found that the excision repair system of bacteria causes few errors in replication, but that the recombination repair system was responsible for nearly all errors in replicated DNA. Since DNA replication is necessary to fix the mutations, the Salmonella tester strains are given enough nutrients to allow several generations of growth during mutagenicity testing.

5. Addition of mammalian liver microsomes to the test system has allowed for the detection of promutagens which require enzyme activation to exhibit mutagenic activity. Bacteria do not possess the enzymes necessary to activate some chemicals to mutagens, so rat or mouse liver microsomal enzymes are generally used

for this purpose. Microsomal preparations of other tissues, such as lung and kidney, may be used to activate chemicals, but they usually contain lower concentrations of the necessary enzymes than liver microsomes (192). The microsomal preparation is incorporated into the mutagenicity test system along with the tester strain and the chemical being tested for mutagenic activity.

Ames, et al. (10) found 18 chemicals that required metabolic activation to induce frameshift mutations in the Salmonella tester strains. Liver microsomes are especially effective for detecting mutagens with planar ring structures. Epoxide derivatives of these chemicals are formed by the enzymes, allowing the mutagens to intercalate between base pairs and bind covalently to the DNA to cause frameshift mutations (11, 14, 238). The mutagenic potency of an intercalating agent is increased 10-100 times if it contains a side chain capable of reacting covalently with DNA (11). Some nitrosamines also require enzyme activation to be detected as mutagens by the tester strains (226).

The microsomal enzymes are associated with the endoplasmic reticulum of the cells. Live animals are induced with phenobarbital, 3-methylcholanthrene or polychlorinated biphenyl mixtures to increase the level of enzymes in the liver capable of activating promutagens to mutagens. Aromatic hydrocarbon hydroxylases (mixed function oxidases) are responsible for the activation of polynuclear aromatic hydrocarbons to epoxides (270). NADPH, flavoproteins and cytochrome P-450 are also involved in epoxide formation. Increased cytochrome P-450 levels have been correlated with increased monooxygenase activity in liver after induction (121).

Since bacteria do not possess the same enzymes as mammals for mutagen activation, many potentially mutagenic chemicals are missed by the Salmonella mutagenicity test if liver microsomes are not used (8). The superiority of a combined bacterial-microsome test system for mutagen screening has been confirmed in comparative tests (46).

6. The sensitivity of the Salmonella microsome/mutagenicity test has been further increased by introducing R-factor plasmids into some of the tester strains. Salmonella tester strains TA 100 and TA 98 are derived from tester strains TA 1535 and TA 1538, respectively, by incorporation of the ampicillin resistance-factor plasmid pKM101 (238). The presence of the R-factor in these strains increases their sensitivity to some mutagens, but not others. The mechanism by which the R-factor increases the sensitivity of the strains to certain mutagens is not clear. Mutagens not readily detected by strains without R-factors, such as aflatoxin B_1, sterigmatocystin, benzo(a)pyrene, nitrofuran and furlyfuramide, are easily detected by the strains with R-factors (13). The addition of R-factor plasmids to the tester strains has greatly extended the range of mutagens detected by the Salmonella mutagenicity test.

A large number of chemicals have been screened by the Salmonella/microsome mutagenicity test and the data base is considerably larger for this test than for other short-term mutagenicity screening tests (131). McCann, et al. (236, 237) assayed over 300 chemicals for mutagenic activity with this test and found a correlation of 87.7 per cent between carcinogenicity and mutagenicity for known carcinogens. Of the known non-carcinogens tested, 86.3 per cent were not mutagenic to the Salmonella tester strains. Ames, et al. (10, 11, 14) have found that most of the carcinogens tested by the Salmonella mutagenicity test are mutagenic, but that many mutagens are not carcinogenic.

Certain classes of chemicals have a higher probability of being mutagenic and/or carcinogenic than others. Aromatic amines, alkyl halides, polynuclear aromatics, esters, epoxides, carbamates, nitro aromatics, heterocyclics, nitrosamines, fungal toxins, antibiotics, cigarette smoke condensates, azo dyes and diazo compounds are especially active in biological systems. Some chemicals may cause frameshift or base-pair substitution mutations in the Salmonella tester strains, exclusively, while others may cause both types of mutation. Chemical classes which cause base-pair substitution mutations, only, in the tester strains include esters, epoxides, carbamates, nitrosamines and miscellaneous nitrogen compounds (237). Polynuclear aromatics, aromatic amines and nitro aromatics commonly cause both types of mutation. No chemical classes are exclusive for frameshift mutations, but individual compounds within the classes may be specific for frameshift mutations. Of 157 carcinogens giving a positive mutagenicity test, 45.2 per cent induced base-pair substitution mutations, 14.8 per cent induced frameshift mutations and 40.0 per cent induced both types of mutation. Overlap in the type of mutation induced by chemicals is common in the Salmonella mutagenicity test (46,237).

The Ames test is quite simple to perform, giving it a decided advantage over many of the short-term screening tests. The test procedure consists of adding the tester strain to melted soft agar along with the chemical being tested and the liver microsomes and overlaying the mixture onto a bottom agar plate. The soft agar contains a small amount of histidine and biotin to allow 3-4 generations of growth of the Salmonella tester strains, which require both histidine and biotin for growth. The bottom agar contains mineral salts and glucose to supply nutrients for the soft agar overlay. The plates are incubated for 48 hours at 37 C and the visible colonies are counted and compared to control plates for an increase in the number of histidine revertants.

The mutants which are not reverted grow as a visible lawn in the clear soft agar. If the soft agar remains clear after 48 hours, the chemical is considered too toxic to the bacteria at that concentration and is tested at a lower concentration. The histidine revertants grow as individual colonies on the surface of the soft agar. A small number of histidine revertant colonies appear spontaneously on control plates. The liver microsome preparation is optional and is not required to detect mutagens that do not need enzyme activation. The microsomal enzymes are active in the soft agar overlay for about six hours at 37 C.

Ames (10) summarized the advantages of the *Salmonella* mutagenicity test as follows:

1. Specific point mutations are used to detect mutagens.
2. A relatively small genome (about 4×10^6 base pairs) is tested.
3. A large number of microorganisms (about 10^9 cells) are exposed to the mutagen.
4. A positive selection for mutated bacterial cells is used.
5. Excision repair is deficient in the tester strains.
6. Lipopolysaccharide is deficient in the tester strains.
7. Mutants are scored in mutational "hot spots."
8. The test has high sensitivity, since only a small fraction of the total mutational events are detected, but nanogram concentrations of mutagens can be detected by the tester strains.

Practical Uses of the Ames Test

Several variations of the Ames test have been used for mutagen detection. Malling (226) devised a liquid test system to detect the mutagenic activity of dimethylnitrosamine, which is not detected on agar plates because of its poor diffusion in agar. This method is not suitable for general mutagenicity testing, however. Legator and Malling (209) developed the host-mediated assay to activate chemicals to mutagens. The *Salmonella* tester strains were injected into the peritoneum of mice and the mutagen was injected into the blood stream to allow *in vivo* metabolic activation of the chemicals tested. The tester strains were recovered from the peritoneum and plated on histidine-free medium to detect histidine revertants. Legator, et al. (208) used the host-mediated assay to study the mutagenic activation of chemicals used for parasite control in animals. *In vivo* tests are difficult to perform and have been largely replaced by liver microsome activation directly on agar plates. Durston and Ames (107) used the plate incorporation assay to detect the mutagenic activation of 2-acetylaminofluorene in rats by feeding the chemical to the rats and testing urine concentrates for mutagenic activity in the plate test. The activated form of 2-acetylaminofluorene was excreted as a *beta*-glucuronide conjugate.

Although the Ames test was designed to test pure chemicals for mutagenic activity, it has been used successfully to test complex chemical substances as well (131). Szybalski (322) claimed that back mutation of E. coli from streptomycin dependence to streptomycin independence could be used to test the mutagenic activity of complex industrial wastes, fermentation liquors, natural products and mold contaminants. Kier, et al. (192) used the Ames test to detect mutagenic activity in cigarette smoke condensates. Rubin, et al. (288) tested fractions of synthetic crude oil derived from coal tar for mutagenic activity with the Ames test and found that the fractions containing polynuclear aromatic hydrocarbons with 4-7 rings had the most mutagenic activity.

The Ames test has been used to detect mutagenic agents in water. Kopfler, et al. (203) concentrated drinking water by reverse osmosis for mutagenicity testing by the Ames test. The concentrates were mutagenic to Salmonella tester strains TA 100 and TA 98. Of 130 chemicals isolated from drinking water, 15 were mutagenic in the Ames test (303). Payne, et al. (270) showed that used crankcase oils were mutagenic when activated by trout liver microsomes and suggested that ingestion of oil by aquatic organisms may increase the mutagenic burden in water. The Ames test was used to detect mutagenic activity in Mississippi River water near New Orleans, Louisiana by Pelon, et al. (271). The highest level of mutagenic activity was detected in water samples obtained near oil and chemical refineries. Seasonal variations in the mutagenic activity of the water was noted also.

Problems with the Ames Test

The suggestion that the mutagenicity of chemicals to bacteria is related to their carcinogenicity to animals has lead to controversy. This idea is based on the somatic theory of carcinogenesis, which states that mutational events in the DNA of mammalian cells are a possible cause of cancer (9, 10). This hypothesis proposes that the DNA composition of bacteria and mammals is similar and that carcinogens are able to cause DNA damage in both systems. The somatic theory of carcinogenesis does not explain some of the other theories of cancer induction, such as viral agents and immune supression mechanisms. The mutational sites in bacteria are different from those which presumably cause cancer in mammals and their enzyme systems do not always produce the same intermediate products from the same substrates. Because bacteria do not get cancer like mammals, the correlation between mutagenicity and carcinogenicity is difficult to prove, conclusively.

The use of short-term screening tests has been proposed as a means of enforcing restrictions on potentially hazardous chemicals. No single test is adequate for this purpose and a battery of short-term screening tests, including procaryotic and eucaryotic test systems is favored to verify the mutagenicity of a chemical. Agreement among short-term screening tests is not perfected yet, but is becoming increasingly better as the tests are improved (131). The link between carcinogenesis and mutagenesis is fairly well established, but extrapolation from mutagenesis in short-term screening tests to carcinogenesis in mammals must be done with caution. Sobel (310) stated that four phases must be evaluated before conclusions can be drawn about the carcinogenic hazard of a chemical on the basis of short-term screening tests:

1. Primary identification or detection of mutagenic activity by short-term screening tests.
2. Verification of mutagenic or carcinogenic activity by other test systems, such as animal testing.
3. Quantification of effects to establish a dose response.
4. Extrapolation to man.

Most of the short-term screening tests lack the natural detoxification mechanisms present in whole animals and the minimum dose causing a mutation in a screening test may be much lower than that required to cause cancer in animals. The relationship between dose response in short-term screening tests and in animals for various chemicals must be established before the screening tests will be of value for enforcement purposes (131).

The type of microsomes and enzyme inducers used affects the sensitivity of the Ames test to certain mutagens. Liver microsomes have the highest concentration of enzymes capable of activating promutagens to mutagens and are used most widely for mutagen activation, although lung and kidney microsomes are also active (192). Gold, et al. (144) compared the ability of rat and mouse liver microsomes to activate a flame retardant to a mutagen when induced with different chemicals. Phenobarbital-induced mouse liver microsomes were weakly active, polychlorinated biphenyl (PCB)-induced rat liver microsomes were weakly active and PCB-induced mouse liver microsomes had strong enzyme activity. Ames (13) recommended the use of rat liver microsomes induced with the PCB mixture, Arochlor 1254. This enzyme preparation is capable of detecting nearly 90 per cent of a series of known carcinogens as mutagens.

The preparation of enzymes for mutagen activation may vary widely from one laboratory to another, causing discrepancies in results. The concentration of enzymes in liver microsome preparations are critical to the sensitivity of the Ames test, as too little or too much will reduce the histidine revertant responses of the tester strains to certain chemicals. Greim, et al. (150) found that protein or choline deficient diets decreased the activity of mouse liver microsomes and that inducers increased their activity. The mutagenic activity of nitrosoguanidine is decreased by mouse liver microsomes, but the activity of dimethylnitrosamine is increased by them.

Since mutagens have a wide range of activity, some do not respond to a given concentration of microsomal enzymes (144).

False positive and false negative responses may occur in the Ames test or other mutagenicity screening tests. The mechanism by which the R-factor plasmids increase the sensitivity of the Ames test is not known. If a test system is too sensitive, it may respond to chemicals that are normally not mutagenic. The use of liver microsome activation in the Ames test has reduced the number of false negative tests, considerably. As the test systems are improved and more data becomes available, the number of false tests are being reduced (46).

Media Selection

The media and methods used for the enumeration of bacteria influence their recovery from aquatic environments. Although Standard Methods (6) currently recommends the use of pour plates for enumerating heterotrophic bacteria in water, Klein (200) demonstrated that environmentally stressed bacteria in water were killed by the transient warming stress of melted agar in pour plates. He recommended the use of spread plates for maximum recovery of heterotrophic bacteria from water. Buck and Cleverdon (51) noted the superiority of spread plates to pour plates for the recovery of marine bacteria.

Harris (154) reviewed some of the factors influencing the recovery of bacteria on various media. The kind of microorganisms recovered and the type of environmental damage they had suffered affected the choice of medium for optimum recovery. Bacteria damaged by freezing or phenolic bacteriocides were recovered best on nutrient-rich media. Irradiation damaged bacteria have been recovered equally well on nutrient-rich and nutrient-poor media. No consensus has been reached as to the best media for their recovery.

Media with low nutrient levels appear to be best for the initial recovery of bacteria from aquatic environments. Schindler, et al. (300) found that a low nutrient medium was best for the recovery of bacteria from unpolluted water, but found little difference in the recovery of bacteria from polluted water on high or low nutrient media. Litchfield, et al. (216) recommended that the nutrient levels of rich media should be lowered to as little as one-tenth that of normal for maximum recovery of bacteria from marine sediments. They were able to recover up to one log unit more bacteria on low nutrient media than on high nutrient media and noted differences in the pigmentation of bacterial colonies as the nutrient levels of the media were changed. Jones (185) compared several media formulations for recovery of bacteria from water and discovered that the inorganic components of a medium had a greater influence on bacterial recovery than the organic components. Addition of lake water or humic substances increased the recovery of bacteria from water, but the results were inconsistent.

The incubation temperature affects the recovery of bacteria from water. Jones (185) found that surface spread plates incubated at 10 C yielded the highest counts of freshwater bacteria. Walker and Colwell (349) reported that the greatest diversity of microbial genera was recovered from oil degradation flasks at 10 C.

A large number of selective media have been developed to recover coliform bacteria from water and they have been tested extensively for recovery of coliforms from a wide variety of aquatic environments. Carlucci and Pramer (61) compared eosin-methylene blue agar and tergitol 7-triphenyltetrazolium chloride agar for recovery of *Escherichia coli* from seawater and observed the best development of colonies on tergitol 7-triphenyltetrazolium chloride agar. Nutting, et al. (262) obtained better recovery of coliforms from ice cream on desoxycholate lactose agar than on violet red bile agar. McBee and McBee (235) used desoxycholate lactose agar to enumerate coliform bacteria in arctic soil and water. Bissonette, et al. (36) compared several methods for recovery of coliforms from a stream. They obtained the poorest recovery on membranes with m-FC broth and m-endo-MF broth. Better recovery was obtained on desoxycholate lactose agar and violet red bile agar pour plates, but recovery efficiency was lost when the coliforms were environmentally stressed. The MPN method, using tubes of brilliant green bile broth, was superior to the other methods. Mossell (252) incubated tergitol 7-triphenyltetrazolium chloride agar at an elevated temperature of 44 C to separate _E. coli_ from high numbers of background contaminants in food and milk which overgrew the _E. coli_ at 37 C.

MATERIALS AND METHODS

The procedures used for water sampling, bacterial enumeration, oil degradation, oil extraction, mutagenicity testing, crude oil fractionation, crude oil adapted cultures, toxicity studies and data analysis are described in this section.

Water Sampling

Water sampling sites located in areas exposed to chronic pollution from industrial or domestic wastes were chosen for study. Surface grab samples were collected for all studies, but sampling procedures varied slightly for different areas and conditions, as described below.

Colorado Water Samples

Water samples were obtained from a waste treatment lagoon at a sugar beet plant in Johnstown, Colorado for preliminary studies conducted at Colorado State University. The lagoon was chronically polluted with machinery lubricating oil, as evidenced by a persistent oil slick at the sampling site.

Surface grab samples were collected in sterile 250 ml wide-mouth polypropylene bottles attached to a sampling rod. The samples were transported to the laboratory in an ice chest, refrigerated immediately at 4 C and processed within eight hours of collection. Water samples were collected at this site in the spring of 1976.

Alaska Water Samples

Water samples for studies conducted at the Arctic Environmental Research Station in College, Alaska were obtained from an established sampling site on the Chena River downstream from the city of Fairbanks. The site, located at Pike's Landing, was designated the C-100 sampling station in a previous study (89). This site was utilized as an ice bridge for automobile traffic during the winter months. Water samples were collected from January to October, 1977.

Winter water samples were obtained by drilling a hole in the ice over the main river channel with a gasoline-powered ice auger. The water level was high enough so that water filled the hole in the ice and was dipped with a sterile plastic dipper into a sterile five gallon polypropylene carboy. The water temperature was taken at the time of sampling. Water samples were transported to the laboratory within 20 minutes, refrigerated immediately at 4 C and processed within eight hours of collection. Appendix 1 lists the equipment required for winter water sampling.

Summer water samples were obtained by wading into the main river channel and collecting surface grab samples directly into sterile five-gallon carboys. The mouths of the carboys were pointed upstream, away from the point of entry into the river and river flow was sufficient to sweep downstream contamination away from the water being collected. The water temperature was taken at the time of sampling. Water samples were transported to the laboratory within 20 minutes, refrigerated immediately at 4 C and processed within eight hours of collection.

Bacterial Enumeration

Several media were evaluated for recovery of heterotrophic and coliform bacteria from water. The media were evaluated as described below and those with the best recoveries were employed in subsequent testing.

Heterotrophic Bacteria

Three media were evaluated for recovery of heterotrophic bacteria from river water at three temperatures. Plate count agar (Difco Laboratories, Inc., Detroit, Mich.), nutrient agar (Difco) and heterotrophic plate count agar (Environment Canada (112)) were tested for recovery of heterotrophic bacteria at 4, 10 and 20 C. The nutrient composition of the three media is compared in Appendix 2.

The spread plate technique was used to enumerate the heterotrophic bacteria. Approximately 25 ml of each medium was poured into 100 X 15 mm plastic petri plates (LabTek Products, Div. Miles Labs, Inc., Elkhart, Ind.) to allow for drying over long incubation times. Serial tenfold dilutions were made in 9.9 and 99.0 ml dilution blanks of sterile phosphate buffer (1.25 ml/l of a 34.0 g/l stock solution of KH_2PO_4 and 5.0 ml/l of a 50 g/l stock solution of $MgSO_4 \cdot 7H_2O$ in distilled water (6)). Spread plates were inoculated with 0.1 ml of serial tenfold dilutions of river water and the inoculum distributed with sterile glass spreaders. Triplicate spread plates were prepared for each dilution and appropriate dilutions were made to yield counts of 30-300 colonies per plate, as recommended by Standard Methods (6). Separate sets of inoculated plates were incubated at 4, 10 and 20 C and counted daily for three weeks to determine the incubation times needed to obtain maximum counts at each temperature.

Coliform Bacteria

Coliform bacteria in river water were enumerated on 0.45 micron membranes (Millipore Corp., Bedford, Mass.). Triplicate aliquots of 10, 50 and 100 ml of river water were filtered through membranes and the membranes placed on filter pads saturated with two ml of m-Endo-MF broth (Difco) in 60 X 15 mm plastic plates (Falcon Plastics, Cockeysville, Md.). The plates were incubated in a moist chamber for 24 hours at 35 C and counted under a 10 X dissecting scope with fluorescent illumination. Plates with 20-80 coliform colonies were counted, as recommended by Standard Methods (6).

Five differential media were evaluated for recovery of *Escherichia coli* K-12 from mixed and pure cultures. The *E. coli* K-12 was provided by Dr. J. E. Ogg, Colorado State University, Fort Collins, Colorado. Eosin-methylene blue agar (Baltimore Biological Laboratories (BBL), Cockeysville, Md.), desoxycholate agar (BBL), tergitol 7-triphenyltetrazolium chloride agar (Difco), brilliant green bile agar (Difco) and violet red bile agar (Difco) were evaluated. Two to four serial tenfold dilutions of bacteria were made in sterile phosphate buffer and 0.1 ml of each dilution was plated in triplicate on spread plates of each medium to yield coliform counts of 30-300 colonies per plate. Inoculated plates were incubated for 24 hours at 35 C and coliform colonies with typical color reactions on the differential media were counted.

When background counts of non-coliform heterotrophic bacteria exceeded coliform counts, an additional set of plates was incubated at 45 C. The elevated temperature decreased the number of non-coliform counts on the differential media substantially without significantly reducing the number of coliform counts.

Oil Degradation

Crude oil degradation studies were performed in 125 or 250 ml Erlenmyer flasks to which 50 or 100 ml of growth medium was added, respectively. The growth medium consisted of sterile salts in distilled water, raw river water or river water supplemented with inorganic salts. Crude oil was added to the flasks in concentrations ranging from 0-5 per cent (w/v). A crude oil concentration of one per cent was routinely tested throughout the study. The flasks were plugged with cotton stoppers to allow gas exchange and aeration. Inoculated test flasks and uninoculated control flasks were incubated in triplicate for 1-6 weeks at temperatures of 4, 20 and 25 C with shaking at 100 rpm on a rotary platform shaker with a one-inch eccentric. Inocula for test flasks consisted of undiluted river water, 1:100 dilutions of river water and 1:100 dilutions of crude oil adapted cultures of microorganisms derived from river water.

The inorganic salts used in the growth medium were modified from those used by Walker and Colwell (349) for low temperature oil degradation studies with estuarine bacteria by lowering the sodium chloride content for freshwater studies (Appendix 3). Salts-supplemented river water was prepared by autoclaving the salts in a minimum of liquid and adding the appropriate volume of river water to the sterile salts. The phosphates were autoclaved separately from the other salts to minimize precipitation during autoclaving. River water used in control flasks was autoclaved and cooled prior to addition of the salts.

Black Hollow crude oil (Weld County, Colorado) was used as the test substrate with Colorado water samples and Prudhoe Bay, Alaska crude oil was used as the test substrate with Alaska water samples. The crude oil was autoclaved in sealed 10 ml glass ampules for 15 minutes at 121 C before use.

Oil Extraction

The crude oil residue remaining after incubation was extracted from the aqueous growth medium by a modification of the gravimetric method for quantitative determination of oil and grease in water described in Standard Methods (6). The contents of a flask to be extracted were acidified with 0.5 ml concentrated HCl, transferred to a 250 ml separatory funnel and extracted three times with 30 ml aliquots of 1,1,2-trichloro-1,2,2-trifluoroethane (Freon^{TM}113, E. I. duPont de Nemours and Co., Inc., Wilmington, Del.). The flask was rinsed with the solvent before each extraction and the solvent was added to the separatory funnel which was shaken vigorously for two minutes. The aqueous and organic phases were allowed to separate and the bottom layer was filtered through Whatman #40 filter paper containing one gram anhydrous sodium sulfate (J. T. Baker Chemical Co., Phillipsburg, N. J.) to dry the extracts and break persistent water-in-oil emulsions. The three extracts were pooled in a tared 300 ml round bottom boiling flask. Prior to the final extraction, 80 ml absolute ethanol (pesticide grade, Fisher

Scientific Co., Waltham, Mass.) was added to the separatory funnel to break oil-in-water emulsions.

The solvent was removed from the oil residue by placing the boiling flask in a 70 C water bath at atmospheric pressure and applying vacuum to the flask for one minute after all visible solvent had evaporated. The evaporated solvent was collected in a condenser tube for re-use as recommended by Anderson (15). The boiling flask containing the crude oil residue was placed in a desiccator jar overnight and weighed on an analytical balance (Mettler H-6) to the nearest 0.1 milligram. Weighed residues from triplicate flasks were re-dissolved in Freon^{TM}113 and combined into one flask for mutagenicity testing. The solvent was removed as described above.

Mutagenicity Testing

The crude oil residue from pooled triplicate extracts was weighed into a pentane rinsed scintillation vial, shaken with two ml dimethyl sulfoxide (DMSO) (spectrograde, Matheson Coleman and Bell Manufacturing Chemists, Norwood, Ohio) and allowed to stand overnight at room temperature. The amount of oil residue weighed into the vial was equivalent to the weight of oil recovered from one gram of fresh crude oil initially added to the incubation flask after extraction with Freon^{TM}113. The weight of oil tested for mutagenic activity ranged from 0.5-0.75 grams. Since only a small portion of the oil residue dissolved in the DMSO, the actual weight of oil tested was unknown.

The Salmonella mutagenicity test described by Ames (13) was used to assay for mutagenic activity in DMSO extracts of the crude oil residues. The five Salmonella tester strains recommended by Ames for general mutagenicity testing were used for all testing: TA 100, TA 1535, TA 1537, TA 1538 and TA 98 (13). The characteristics of the tester strains are listed in Appendix 4. Mammalian liver microsomes were not employed in the mutagenicity testing of crude oil residues.

A soft agar overlay technique was used to test the crude oil residues for mutagenic activity. The bottom agar consisted of Vogel-Bonner E salts (344), 2.0 per cent glucose (Difco) and 1.5 per cent agar (Difco). Thirty ml of the sterile, melted medium was poured into each 100 X 15 mm plastic petri plate (LabTek Products, Div. Miles Labs, Inc., Elkhart, Ind.) and allowed to solidify. The bottom agar composition and preparation is shown in Appendix 5.

The top agar overlay consisted of 0.6 per cent agar (Difco) and 0.5 per cent sodium chloride per liter of distilled water. The top agar was sterilized in dilution bottles in 100 ml volumes. Before use, the top agar was re-melted in a boiling water bath and 10 ml of a sterile solution of 0.5 mM L-histidine·HCl (Sigma Chemical Co., St. Louis, Mo.) and 0.5 mM d-biotin (Sigma) in distilled water was added and mixed by inversion. Two ml aliquots of the top agar were dispensed into capped, sterile disposable 13 X 150 mm glass test tubes (Corning Glass Works, Corning, New York) in a 45 C water bath.

To the melted top agar in a test tube, 0.1 ml of the DMSO extract of a crude oil residue and 0.1 ml of the appropriate Salmonella tester strain culture were added and the contents shaken on a vortex mixer. The top agar was rapidly transferred to the surface of a plate of bottom agar and evenly distributed on the surface. The soft agar overlay was allowed to solidify and the plates were incubated, upside-down, in the dark for 48 hours at 35 C. Visible colonies appearing on the plates after 48 hours were counted as histidine revertants. The crude oil residue was plated in triplicate with each Salmonella tester strain along with triplicate control plates.

The Salmonella tester strains were provided by Dr. B. N. Ames, University of California, Berkley, Ca. Upon receipt of the tester strains, they were transferred to five ml nutrient broth (Difco) with 0.5 per cent sodium chloride added and incubated with shaking for 16 hours at 35 C. The strains were tested for appropriate reactions to standard mutagens and for the presence of the proper mutations (Appendices 6 and 7). Each tester strain was preserved by adding 0.8 ml of a nutrient broth culture and 0.07 ml DMSO to a sterile, two ml, rubber-stoppered, screw-capped glass vial, quick-freezing in a Dry Ice-acetone bath and storing in a deep freeze at -80 C.

To prepare the Salmonella tester strains for mutagenicity testing, the surface of each frozen culture was scraped with a sterile wooden applicator stick and inoculated into five ml nutrient broth with 0.5 per cent sodium chloride added. The cultures were incubated in a 37 C water bath with shaking for a maximum of 16 hours. The cultures were stored at 4 C and could be used for one week.

The Salmonella tester strains were periodically tested for appropriate reactions to the standard mutagens, methyl methane sulfonate (ICN Pharmaceuticals, Inc., Cleveland, Ohio), 4-nitroquinoline-N-oxide (ICN), N-methyl-N-nitro-N-nitrosoguanidine (ICN), 9-aminoacridine (Sigma) and daunomycin (Sigma) (13). These mutagens do not require liver microsome activation to be detected by the tester strains. Typical test reactions are shown in Appendix 6.

The stability of the genetic characteristics of the Salmonella tester strains shown in Appendix 4 were tested periodically (13). Presence of the R-factor was determined by ampicillin (BBL) resistance, the lipopolysaccharide deletion was confirmed by crystal violet sensitivity, the excision repair deficiency was determined by ultraviolet light sensitivity, the histidine and biotin requirements were tested on nutrient deficient medium and spontaneous reversion was determined on mutagenicity test plates in the absence of mutagens. Typical test reactions are shown in Appendix 7.

Crude Oil Fractionation

Crude oil was fractionated into saturate, aromatic and nitrogen-sulfur-oxygen (NSO)-containing compounds by column chromatography as described by Jobson, et al. (184). The fractions were tested for mutagenic activity before and after incubation with raw and sterile salts-supplemented Chena River water.

One gram of Prudhoe Bay crude oil was weighed into a tared 150 ml beaker, topped (volatiles removed) for 19 hours at 40 C, cooled in a desiccator jar and reweighed. The topped oil was dissolved in 40 ml/g ± 1 ml/g n-pentane (nano-grade, Mallinkrodt Chemical Works, St. Louis, Mo.) and allowed to stand for two hours to precipitate pentane insoluble asphaltenes. The oil was deasphaltened by passing it through an 11 X 500 mm glass column fitted with a Teflon stopcock which was packed with six grams of diatomaceous earth (HyFlo SuperCel, Fisher Scientific Co., Waltham, Mass.) and pre-wetted with n-pentane. The bottom of the column was plugged with glass wool. The pentane soluble portion of the oil was collected in a 250 ml sidearm flask with water aspiration and the column washed with four successive 15 ml volumes of n-pentane. Benzene soluble asphaltenes were eluted from the column into a clean 250 ml sidearm flask with four successive 15 ml volumes of benzene (pesticide grade, Fisher). The deasphaltened oil and the benzene soluble asphaltenes were transferred to tared 150 ml beakers and placed in a fume hood overnight to evaporate the solvents. The beakers were placed in a 40 C oven for three hours to remove residual solvent and attain a constant weight. The beakers were cooled in desiccator jars before weighing to the nearest 0.1 milligram. Insoluble asphaltenes were calculated by difference.

Saturate, aromatic and NSO fractions were obtained by dissolving the deasphaltened oil in 10 ml n-pentane and passing it through a 19 X 500 mm glass column fitted with a Teflon stopcock and a 300 ml top reservoir. The column was packed with 13 grams of 20-200 mesh silica gel (Baker) activated at 150 C for 16 hours followed by 13 grams of activated alumina (Alcoa F-20, Fisher). The column was filled with n-pentane and packed slowly with agitation to remove bubbles from the column. The bottom of the column was plugged with glass wool.

The saturate fraction was eluted from the column with 240 ml n-pentane and collected in a tared 400 ml beaker. The aromatic fraction was eluted next with 300 ml benzene. The NSO fraction was eluted last with 300 ml benzene-methanol (1:1). The beakers were placed in a fume hood overnight to evaporate the solvents, placed in a 40 C oven for three hours to remove residual solvents and cooled in desiccator jars before weighing to the nearest 0.1 milligram. Insoluble NSO's were calculated by difference.

Crude Oil Adapted Cultures

All mutagenicity and toxicity studies conducted at Colorado State University utilized crude oil adapted cultures. The adapted cultures were established by diluting water samples 1:100 in sterile salts medium and adding one per cent sterile Black Hollow crude oil as the carbon and energy source to enrich for oil-degrading microorganisms. The adapted cultures were incubated in flasks for 1-3 weeks at 25 C and maintained by successive transfers of 1:100 dilutions of the growth to fresh salts medium and crude oil.

The majority of the tests conducted at College, Alaska used fresh river water samples as the inoculum for test flasks, but a few tests were performed with Prudhoe Bay crude oil adapted microbial cultures in an attempt to stabilize the test system. These cultures were established as described above using autoclaved river water supplemented with salts and incubation temperatures of 4 and 20 C.

Toxicity Studies

The toxicity of crude oil to the non-oil-degrading bacterium, *Escherichia coli* K-12, was determined in the presence and absence of oil-degrading microorganisms by monitoring the decline of the

E. coli K-12 population on a selective medium. Four 250 ml Erlenmyer flasks with 100 ml volumes of medium were initially inoculated with 10^6-10^7 cells per ml of E. coli K-12 to test the effects of Chena River water, oil and naturally-occurring microorganisms on the survival of the E. coli K-12 population. The flasks contained the following media: 1) sterile, salts-supplemented river water; 2) raw, salts-supplemented river water; 3) sterile, salts-supplemented river water with one per cent Prudhoe Bay crude oil; and 4) raw, salts-supplemented river water with one per cent Prudhoe Bay crude oil.

The flasks were incubated for one week at 20 C with shaking at 100 rpm. One ml samples were taken from the flasks at 12-24 hour intervals and diluted in sterile phosphate buffer. One-tenth ml of each dilution was plated in triplicate on violet red bile agar plates and typical, red coliform colonies were counted after incubation for 24 hours at 35 C. Growth of heterotrophic oil-degrading bacteria in the flasks was monitored on triplicate plates of heterotrophic plate count agar (HPCA) incubated at 4 C and counted after three weeks. Since the E. coli K-12 did not grow at 4 C, it was possible to enumerate the non-coliform heterotrophic bacteria from the river water separately from the E. coli K-12. Shaw, et al. (296) determined that the minimal growth temperature of a cold tolerant E. coli strain was 7.5-7.8 C.

Crude oil adapted cultures were also used for toxicity studies with *E. coli* K-12. Cultures containing about 10^8 cells/ml were diluted 1:10,000 in sterile river water supplemented with salts to yield an initial cell density of approximately 10^4 cells/ml, similar to the density of heterotrophic bacteria in raw river water.

A predator-prey relationship, which caused a rapid decline in the *E. coli* K-12 population, was observed when raw Chena river water was used as the inoculum for toxicity studies. This phenomenon was studied by a soft agar overlay technique. One-tenth ml of an overnight culture of *E. coli* K-12, grown in nutrient broth at 35 C, and 0.1 ml of a river water culture exhibiting toxicity to *E. coli* in the absence of crude oil, were mixed in a test tube with two ml melted soft agar (0.5 %) at 45 C. The soft agar was overlaid on the surface of a plate of nutrient agar. A control plate was inoculated with *E. coli* K-12 and a crude oil adapted culture of microorganisms that were not toxic to the *E. coli* K-12. The plates were incubated at 30 C and observed daily for clear zones in the lawn of bacteria. Clear zones were observed microscopically under a high-dry objective.

An enrichment culture technique was used to further study the agent responsible for the rapid decline of *E. coli* K-12 in raw river water. Two tubes of *E. coli* K-12 grown in ten ml nutrient broth overnight at 35 C were washed three times in phosphate buffer and resuspended in ten ml phosphate buffer to obtain turbid cell suspensions. One-tenth ml of a river water culture exhibiting toxicity to *E. coli* K-12 was added to one of the tubes and the other tube was used as a control. The suspensions were incubated at room temperature and observed daily for clearing. When clearing was detected, the suspensions were examined microscopically in wet mount preparations.

Data Analysis

Mutagenic activity of crude oil was expressed as a ratio of the average number of *Salmonella* histidine revertants on triplicate test plates to the average number of revertants on triplicate control plates. Total, non-biologically-mediated and microbiologically-mediated mutagenic activities in the crude oil residues were calculated as follows:

$$\text{Total mutagenic activity} = \frac{\text{Avg. \# histidine revertants/test oil}}{\text{Avg. \# histidine revertants/solvent control}}$$

$$\text{Non-biologically-mediated mutagenic activity} = \frac{\text{Avg. \# histidine revertants/control oil}}{\text{Avg. \# histidine revertants/solvent control}}$$

$$\text{Microbiologically-mediated mutagenic activity} = \frac{\text{Avg. \# histidine revertants/test oil}}{\text{Avg. \# histidine revertants/control oil}}$$

The total mutagenic activity of test oil extracts consisted of the non-biologically-mediated and microbiologically-mediated mutagenic activity present in the oil after incubation in river water containing oil-degrading microorganisms. The non-biologically-mediated mutagenic activity was determined in a control oil which was incubated in sterile river water for the same length of time as the test oil. The total and non-biologically-mediated mutagenic activity of test and control oil extracts was expressed as the ratio of the average number of histidine revertants on triplicate mutagenicity test plates containing test and control oil extracts, respectively, to the average number of histidine revertants on solvent control plates without oil. Microbiologically-mediated mutagenic activity was determined in a test oil after incubation with microorganisms and was expressed as the average number of histidine revertants on triplicate mutagenicity test plates containing a test oil extract to the average number of histidine revertants on mutagenicity test plates containing a control oil extract. As long as the microbiologically-mediated mutagenic activity of the test oil extract was significantly greater than the non-biologically-mediated mutagenic activity of the control oil extract, the relative levels of mutagens formed by these two processes could be determined.

Three sets of control plates were run in triplicate for each *Salmonella* tester strain with each mutagenicity test of microbiologically-degraded oil. A strain control, containing no mutagens or other additions, was used to determine the normal number of spontaneous background reversions for each Salmonella tester strain. A solvent control, containing 0.1 ml dimethyl sulfoxide, was used to determine total and non-biologically-mediated mutagenic activity in test and control oil extracts. Although the strain and solvent controls served essentially the same purpose in the Ames test, the solvent control was used for determining histidine revertant ratios, since the test and control oil extracts were dissolved in dimethyl sulfoxide.

Ames (13) recommended that a mutagenicity test plate should have at least twice as many histidine revertants as the control plate before a chemical is considered mutagenic in the Salmonella mutagenicity test. Oil extracts yielding histidine revertant ratios of 2.0 or more were considered mutagenic on this basis. To test the validity of this assumption, standard deviations of the average number of histidine revertants on 29 sets of strain and solvent controls plated in triplicate were calculated to determine the confidence interval within which a doubling of the mean occurred for each tester strain (309). These controls were run at different times throughout the study and were considered representative of the variability expected among control plates. The results of this study are shown in Table 21.

A paired t test was performed on the average number of histidine revertants on 29 matched sets of strain and solvent controls to determine if there was any significant difference between the two controls (309). This test was run to see if there was any advantage of using one set of controls over the other for determining histidine revertant ratios. The results of this study are shown in Table 21.

The dose response curves in Figure 10 were tested for linearity by linear regression analysis (309). The dose response curves were plotted in the manner described by Ames (13). Dose response curves with linear correlation coefficients of 0.95 or greater were considered to have good linearity.

RESULTS

The results of media evaluation, oil degradation, toxicity studies, mutagenicity testing and data analysis are presented in this section.

Media Evaluation

The results of media evaluation tests for optimal recovery of heterotrophic and coliform bacteria from water are given below. These results were used to select media for subsequent studies of oil degradation and the toxicity and mutagenicity of oil.

Enumeration of Heterotrophic Bacteria

Three media were evaluated for initial recovery of heterotrophic bacteria from Chena River water: nutrient agar (NA), plate count agar (PCA) and heterotrophic plate count agar (HPCA). The average number of bacterial colonies recovered on triplicate spread plates of each medium inoculated with 0.1 ml of a 10^{-2} dilution of Chena River water and incubated at 10 C are compared in Figure 2. HPCA recovered the highest number of heterotrophic bacteria from river water under these conditions.

Differences in the morphology and pigmentation of bacterial colonies were evident on each medium. Colonies were larger on

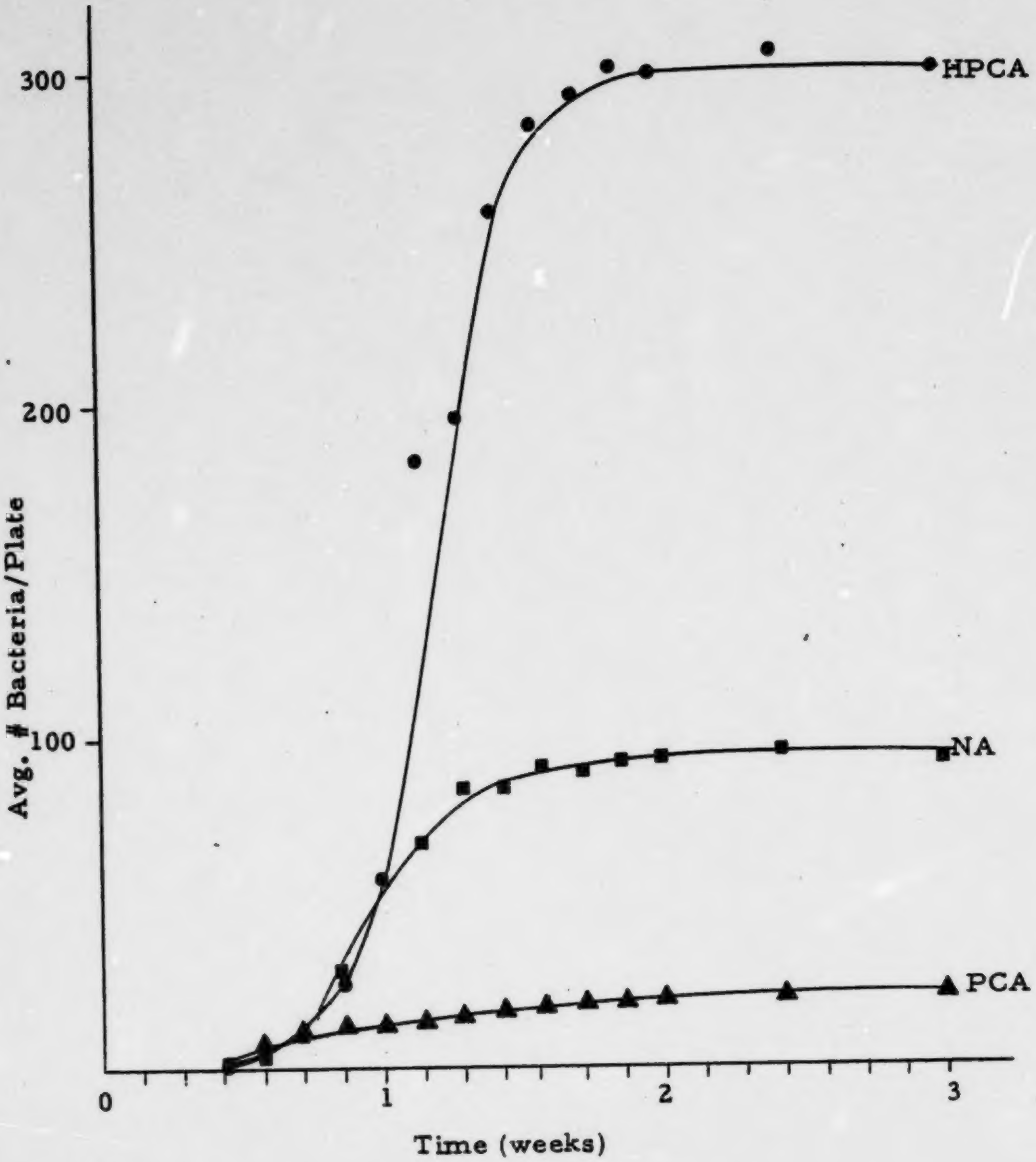

Figure 2. Comparison of the average number of heterotrophic bacteria recovered from a 10^{-3} dilution of a March Chena River water sample on triplicate spread plates of three media at 10 C over time and the incubation times required for maximum recovery. ● - heterotrophic plate count agar (HPCA), ■ - nutrient agar (NA), ▲ - plate count agar (PCA).

PCA than on NA and HPCA and tended to spread and overlap upon prolonged incubation. Colonies on NA and HPCA were small and discrete, except for a few flat, spreading types. Pinpoint colonies that grew on HPCA, but not on NA or PCA, were primarily responsible for the increased recovery of bacteria on HPCA. Pigmentation was expressed most strongly on PCA and least strongly on HPCA. The pigment colors expressed by some bacteria were not uniform on each medium.

To further test the recovery capabilities of the three media, 33 distinct colony types were picked from HPCA plates and inoculated onto grid plates of all three media. Only one colony type failed to grow on HPCA, but five isolates did not grow on NA and 11 isolates did not grow on PCA at 10 C. On the basis of the above results, HPCA was selected for routine enumeration of heterotrophic bacteria from river water and oil degradation flasks.

The temperature of incubation had a slight effect on the recovery of heterotrophic bacteria from river water. The recovery of heterotrophic bacteria from Chena River water on HPCA at 4, 10 and 20 C is shown in Figure 3. An incubation temperature of 10 C yielded the highest bacterial counts. The incubation times required for maximum recovery of heterotrophic bacteria from river water at each temperature were approximately three weeks at 4 C, two weeks at 10 C and one week at 20 C. Figure 2 indicates that the medium

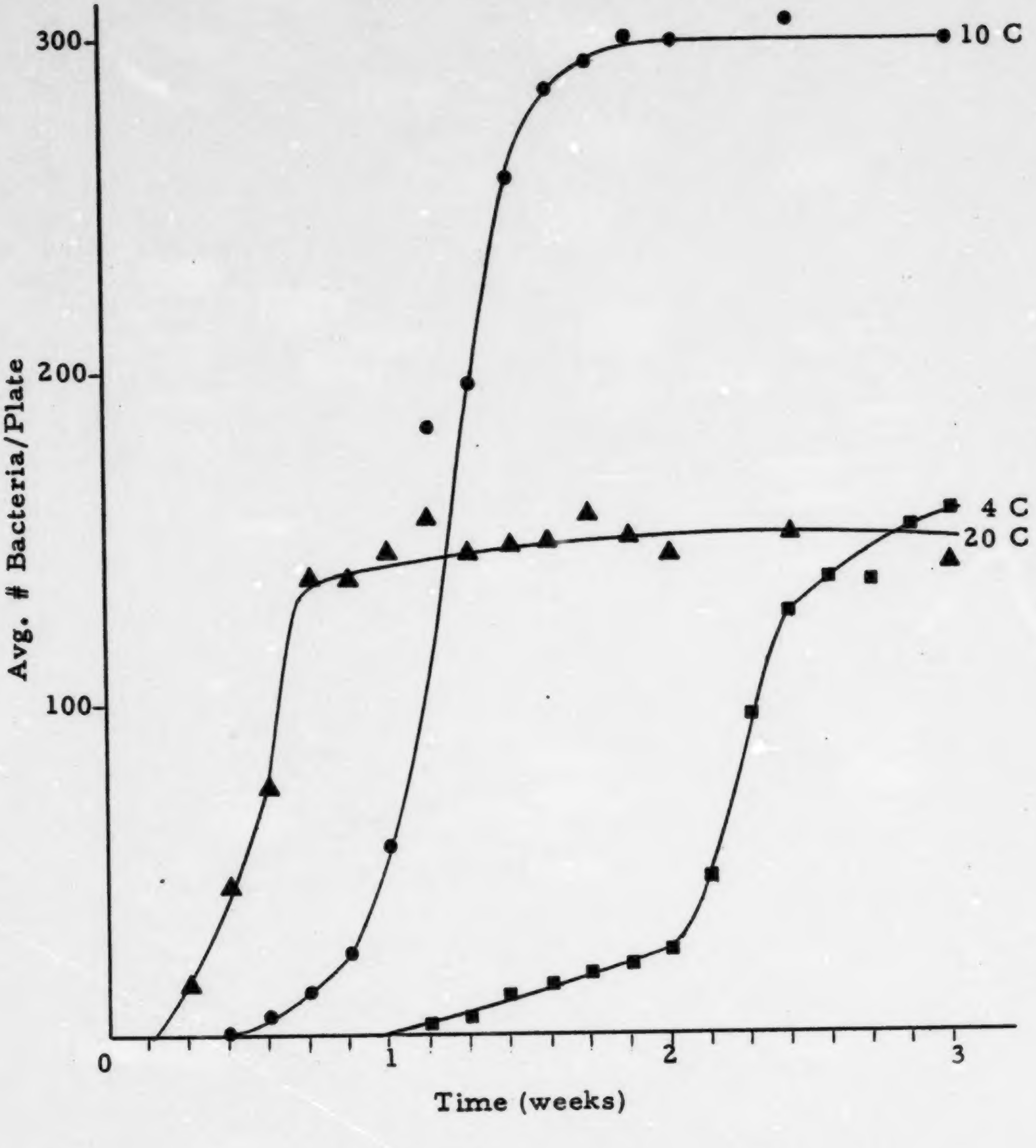

Figure 3. Comparison of the average number of heterotrophic bacteria recovered from a 10^{-3} dilution of a March Chena River water sample on triplicate spread plates of heterotrophic plate count agar (HPCA) at three temperatures over time and the incubation times required for maximum recovery. ● - 10 C, ■ - 4 C, ▲ - 20 C.

composition had little effect on the incubation time required for maximum recovery at 10 C.

Enumeration of Coliform Bacteria

Total coliform counts of Chena River water were obtained to determine the extent of pollution by domestic wastes, since a water sampling site with previous exposure to pollution was desired. Total coliform counts of 100/100 ml of river water were consistently obtained at the C-100 sampling station downstream from the city of Fairbanks. For comparative purposes, a sampling site upstream from the city, designated the C-600 station, was also tested. Total coliform counts at this site were 1/100 ml of river water, indicating relatively greater pollution at the C-100 station.

Five differential media were evaluated for recovery of *Escherichia coli* K-12 from pure and mixed cultures of bacteria in oil toxicity studies: eosin-methylene blue agar, tergitol 7-triphenyltetrazolium chloride agar, brilliant green bile agar, desoxycholate agar and violet red bile agar. All five media recovered comparable numbers of *E. coli* K-12 from pure culture. When the number of non-coliform bacteria exceeded that of *E. coli* K-12 in mixed cultures, the ability of eosin-methylene blue agar, tergitol 7-triphenyltetrazolium chloride agar and brilliant green bile agar to differentiate between coliform and non-coliform bacteria was impaired. The alkaline conditions produced in these media by the non coliform bacteria masked the acid dependent color reactions of the coliform bacteria. Recovery of *E. coli* K-12 from mixed cultures was poorest on brilliant green bile agar.

Desoxycholate agar and violet red bile agar were nearly equal in their ability to differentiate *E. coli* K-12 from heavy background growth of non-coliforms. Coliform colonies were easily distinguished from non-coliforms on these media, even in nearly confluent growth, appearing as red dots in the white background growth. Violet red bile agar was chosen for routine enumeration of *E. coli* K-12 in toxicity studies of oil because of the intensity of its color reaction.

None of the differential media tested were able to substantially reduce the non-coliform bacterial populations when incubated at 35 C. Incubation of violet red bile agar plates at an elevated temperature of 44.5 C reduced the number of non-coliforms in mixed cultures so that the coliforms could develop without excessive crowding. The coliform counts at 35 C and 44.5 C on violet red bile agar were nearly the same, indicating that there was no significant reduction of counts due to the elevated temperature. Recovery of *E. coli* K-12 on nutrient agar was comparable to that of violet red bile agar, showing that the selective components of the differential medium were not inhibitory to *E. coli* K-12. Since no significant depression of *E. coli* K-12 counts was caused by elevated temperatures, overcrowding or selective components on violet red bile agar, this medium was used to study the toxicity of crude oil to E. coli K-12 in the presence of oil-degrading microorganisms.

Oil Degradation Studies

Oil degradation flasks were monitored for heterotrophic bacterial numbers and weight loss of Freon^{TM}-extractable oil under varying temperature and nutrient conditions. Figure 4 shows the effects of temperature and nitrogen and phosphorus salts on the number of heterotrophic bacteria in oil degradation flasks. In the presence of excess nitrogen and phosphorus, the number of heterotrophic bacteria growing on one per cent Prudhoe Bay crude oil was limited by time but not temperature. After four weeks incubation, the number of heterotrophic bacteria growing in salts-supplemented Chena River water with crude oil as the carbon source was nearly the same at 4 and 20 C. Incubation time was the primary limiting factor when nutrient levels were equal and temperature was variable.

Raw Chena River water was not able to support as many heterotrophic bacteria on one per cent Prudhoe Bay crude oil as salts-supplemented river water. Heterotrophic bacterial counts in salts-supplemented river water exceeded 10^8 cells/ml, but raw river water supported only 10^6-10^7 cells/ml at 20 C (Figure 4). These results indicate that Chena River water did not have an adequate supply of non-hydrocarbon nutrients to support maximum bacterial growth on Prudhoe Bay crude oil.

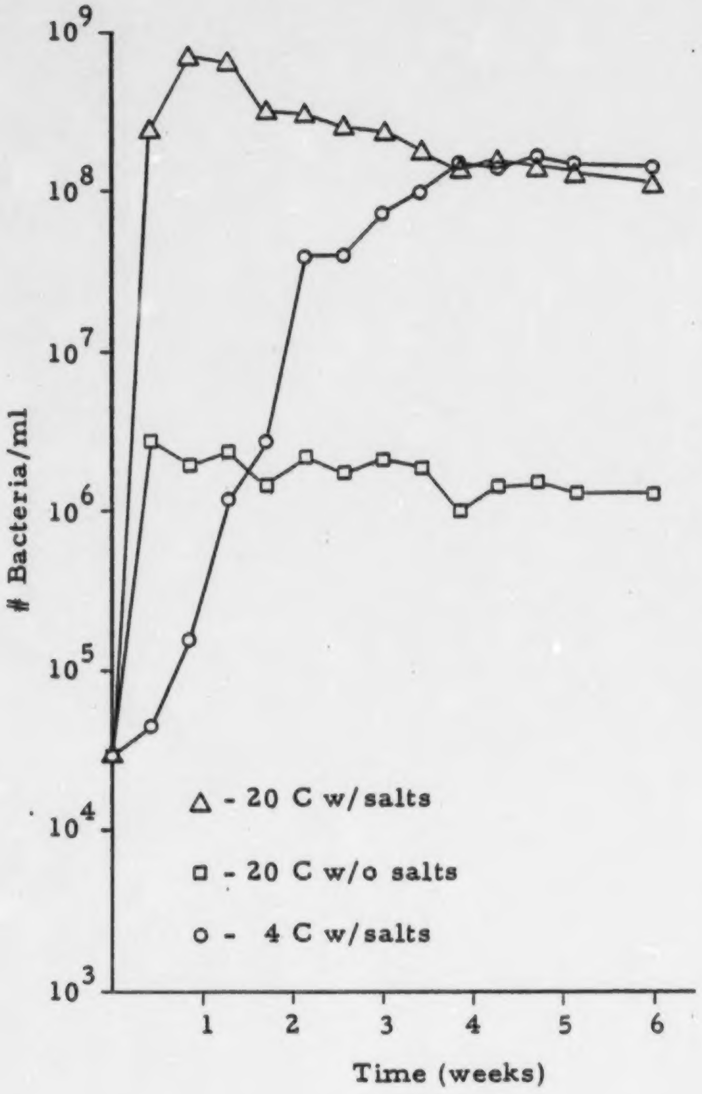

Figure 4. Effects of temperature and inorganic nitrogen and phosphorus salts on the number of heterotrophic bacteria recovered from oil degradation flasks containing March Chena River water samples and 1% Prudhoe Bay crude oil over time on heterotrophic plate count agar (HPCA) incubated for one week at 20 C.

The weight loss of Freon$^{\text{TM}}$-extractable crude oil from oil degradation flasks at 4 and 20 C in raw and salts-supplemented Chena River water over time is shown in Figure 5. The weight loss from sterile control flasks and flasks with raw river water was nearly identical at both temperatures. Non-biological weight losses amounted to 5-8 per cent after six weeks incubation at 4 and 20 C. An initial weight loss of 21.6 per cent at zero time was due to the loss of volatile and Freon$^{\text{TM}}$-insoluble components of the oil during extraction of the oil from water. The remaining 78.4 per cent represented 100 per cent of the Freon$^{\text{TM}}$-extractable components of the oil. The weight loss of oil from flasks with salts-supplemented river water was noticeably greater than from flasks with raw or sterile river water. Biological weight losses amounted to 20 per cent at 20 C and six per cent at 4 C after six weeks.

To determine if the solvent extracted a significant amount of weight from the microbial cells in oil degradation flasks, heavy suspensions of cells were grown in flasks with a non-hydrocarbon substrate of 0.1 per cent vitamin-free casamino acids and salts medium in distilled water. Less than one milligram of residue was extracted from each flask with Freon$^{\text{TM}}$113. The residue extracted from 100 ml of raw Chena River water was also less than one milligram. The oil extraction procedure did not appear to be adversely affected by substances other than the crude oil.

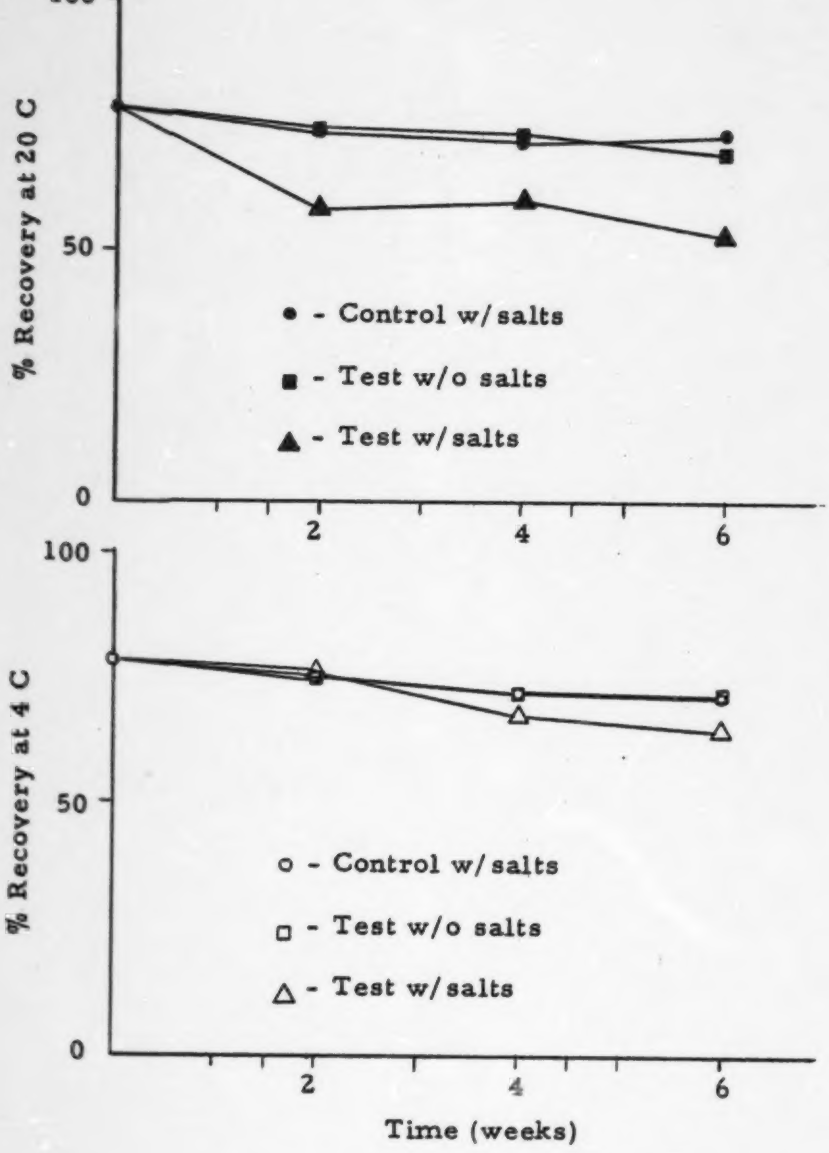

Figure 5. Effects of temperature and inorganic nitrogen and phosphorus salts on the percentage of crude oil recovered from oil degradation flasks containing March Chena River water samples and 1% Prudhoe Bay crude oil over time by Freon^{TM} extraction.

Figures 4 and 5 show that the incubation time required to detect a weight loss of oil due to microbiological degradation corresponds to the beginning of the stationary phase of bacterial growth -- about one week at 20 C and 3-4 weeks at 4 C. They also show that the non-hydrocarbon nutrients in raw Chena River water were insufficient for maximum utilization of crude oil by microorganisms. The inorganic salts supplement added to the river water was sufficient to increase the amount of oil degradation by microorganisms noticeably and support near maximum numbers of heterotrophic bacteria on crude oil. The lack of non-hydrocarbon nutrients in Chena River water appears to be a primary limiting factor in the microbial degradation of oil.

Table 8 lists the levels of nitrogen, phosphorus and carbon in Chena River water at the C-100 sampling station from January to April 1976. Nitrogen and phosphorus levels were generally quite low, usually less than one mg/l. Carbon levels were high enough to support some microbial growth, but any growth of microorganisms in the river water was subject to limitation by the nitrogen and phosphorus levels. An initial increase of approximately two log units in the number of heterotrophic bacteria in raw Chena River water at 20 C can be seen in Figure 4. Flasks of raw river water without crude oil added supported a similar number of heterotrophic bacteria at 20 C, so this increase was mainly due to utilization of nutrients

Table 8. Concentration range of nitrogen, phosphorus and carbon in water samples collected at the C-100 station on the Chena River from Jan. -Apr. 1976.

Parameter	Concentration range mg/l
Total nitrogen	0.51-1.03
Ammonia nitrogen	0.04-0,62
Nitrate nitrogen	0.01-0.26
Nitrite nitrogen	0.001-0.030
Total phosphorus	0.06-0.31
Ortho-phosphate	0.01-0.29
Total carbon	28.0-32.0
Total organic carbon	17.2-21.7

Unpublished data provided by Mr. John Colvin, Arctic Environmental Research Laboratory, U. S. Environmental Protection Agency, College, Alaska.

present in the river water prior to addition of the oil. This observation is supported by Figure 5 which shows that the weight loss of oil by microbial degradation was minimal in raw Chena River water, since the weight loss of Freon^{TM}-extractable oil from these flasks was nearly identical to the weight loss from control flasks due to non-biological weathering processes.

The effect of the initial crude oil concentration in oil degradation flasks on the percentage of Prudhoe Bay crude oil biodegraded in salts-supplemented Chena River water is shown in Table 9. At Prudhoe Bay crude oil concentrations up to 0.5 per cent, the weight loss due to non-biological and biological processes ranged from 49-64 per cent of the total starting weight. According to Figure 5, 29 per cent of the initial weight of oil was lost from sterile control flasks after four weeks at 20 C, indicating that 20-35 per cent of the weight loss was due to biodegradation. The weight of oil biodegraded increased with oil concentration up to one per cent, but the percentage of oil biodegraded began to decline at this level. An initial Prudhoe Bay crude oil concentration of five per cent completely inhibited microbial degradation.

The appearances of the oil and aqueous phases of ten replicate oil degradation flasks after two weeks incubation at 20 C are recorded in Table 10. Visual observation of replicate oil degradation flasks revealed that oil degradation processes were highly variable from

Table 9. Effect of initial Prudhoe Bay crude oil concentration on the amount of oil biodegraded after four weeks incubation at 20 C with salts-supplemented April Chena River water samples.

Initial crude oil concentration		Amount oil lost		Amount oil biodegraded**	
(% w/v)*	(mg)	(mg)	(%)	(mg)	(%)
0	0	0	0	0	0
0.01	5.5	3.2	58.2	1.6	29.2
0.05	27.0	13.2	48.9	5.4	19.9
0.1	54.7	34.8	63.6	18.9	34.6
0.5	250.0	132.0	52.8	59.5	23.8
1.0	500.2	220.8	44.2	76.0	15.2
2.0	1000.2	326.3	32.8	38.0	3.8
5.0	2500.6	708.2	28.3	0	-0.7

*Initial crude oil concentration expressed as per cent (w/v) oil in 50 ml river water.

**The 29 per cent non-biological weight loss of Prudhoe Bay crude oil from control flasks after four weeks incubation at 20 C recorded in Figure 5 was subtracted from amount oil lost to obtain amount oil biodegraded.

Table 10. Appearance of the oil and aqueous phases of ten replicate oil degradation flasks after incubation of 1% Prudhoe Bay crude oil for two weeks at 20 C with salts-supplemented October Chena River water samples.

Replicate	Phase appearance	
	Oil	Aqueous
Control	Intact unbroken slick No emulsion	Clear Colorless
R1	Broken slick Fine flaky emulsion	High turbidity Dark yellow-brown
R2	Broken slick Fine flaky emulsion	High turbidity Dark yellow-brown
R3	Residual scum-no slick Fine flaky emulsion	Moderate turbidity Pale yellow
R4	Broken slick Stringy emulsion	High turbidity Dark brown
R5	Residual scum-no slick Globular emulsion	Slight turbidity Pale yellow
R6	Residual slick Large globular emulsion	Moderate turbidity Yellow-orange
R7	Residual scum-no slick Globular emulsion	Moderate turbidity Yellow
R8	Broken slick Fine flaky emulsion	High turbidity Dark brown
R9	Broken slick Fine globular emulsion	Moderate turbidity Yellow-orange
R10	Residual scum-no slick Fine globular emulsion	Moderate turbidity Yellow-orange

flask to flask. The total number of bacteria recovered from replicate flasks was about the same (greater than 10^8 cells/ml), but the predominant bacterial types were variable. The predominant bacteria in replicate flasks varied with the time and temperature of incubation, nutrient levels, oil concentration and unknown differences in replicate flasks. The most noticeable change was a shift to a lower ratio of pigmented to non-pigmented bacterial types.

The naturally-occurring river water microorganisms were adapted to growth on Prudhoe Bay crude oil in an attempt to eliminate some of the variability among replicate flasks. Visible differences among replicate flasks were reduced, but not completely eliminated, when oil degradation flasks were inoculated with crude oil adapted cultures. It was not possible to stabilize the adapted populations, as shifts in the predominant bacterial types were seen after several successive transfers to fresh crude oil. The adapted cultures were able to emulsify the crude oil more rapidly and completely after several transfers than unadapted microorganisms from fresh river water samples.

Microscopic observation of oil droplets emulsified by microorganisms revealed that most of the droplets had rod-shaped bacteria on their surface. Oil droplets surrounded by heavy visible growth had a layer of bacteria on the immediate surface of the drop which was covered by a thick mat of fungal mycelia, conidia and budding yeasts. The microorganisms appeared to be associated with the oil-water interface and no evidence of penetration of the droplets by microorganisms was seen. All of the bacterial isolates examined were Gram-negative rods.

An attempt was made to study the degradation of crude oil by pure cultures of bacteria. Predominant bacteria from oil degradation flasks were isolated on HPCA streak plates and pure cultures of the isolates were inoculated into flasks containing salts medium in distilled water and one per cent Prudhoe Bay crude oil as the sole carbon source. The pure cultures grew poorly on fresh crude oil, so this phase of the investigation was not pursued.

Toxicity Studies

The acute toxicity of Prudhoe Bay crude oil to the non-oil-degrading bacterium, Escherichia coli K-12, was determined in the presence and absence of oil-degrading microorganisms in salts-supplemented Chena River water. The survival of E. coli K-12 in test and control flasks was monitored on violet red bile agar spread plates and the heterotrophic bacterial numbers were monitored on HPCA spread plates. In the absence of oil-degrading microorganisms, the survival of E. coli K-12 was essentially the same in flasks with and without oil. The flasks were initially inoculated with 10^7 cells/ml of E. coli K-12 and no decrease in numbers was detected in either of these flasks after two weeks at 20 C. Sterilization of the river water by autoclaving or membrane filtration had no effect on the survival of *E. coli* K-12. These flasks established that the river contained no chemicals lethal to the test organism, the fresh crude oil was not lethal to the test organism and the test organism did not grow on the fresh crude oil.

Figure 6 shows the results of a toxicity test using salts-supplemented river water containing the naturally-occurring river water microorganisms. Two test flasks were incubated for two weeks at 20 C; one with oil to test the toxic effect of Prudhoe Bay crude oil on *E. coli* K-12 during oil degradation by the river water microorganisms and one without oil to test the toxic effect of the naturally-occurring river water microorganisms on the *E. coli* K-12 in the absence of oil. An initial increase in the number of *E. coli* K-12 in both flasks was due to utilization of nutrients in the river water. These nutrients were rapidly used within the first day and caused only a slight increase in the number of *E. coli* K-12. After three days of incubation, a rapid decline in the number of *E. coli* K-12 was seen in the flask without oil, indicating that some factor other than crude oil was lethal to *E. coli* K-12 in raw Chena River water. A decline in the number of heterotrophic bacteria in the same flask showed that the lethal effect was not specific for *E. coli* K-12. A more gradual decline of *E. coli* K-12 occurred in the flask with oil

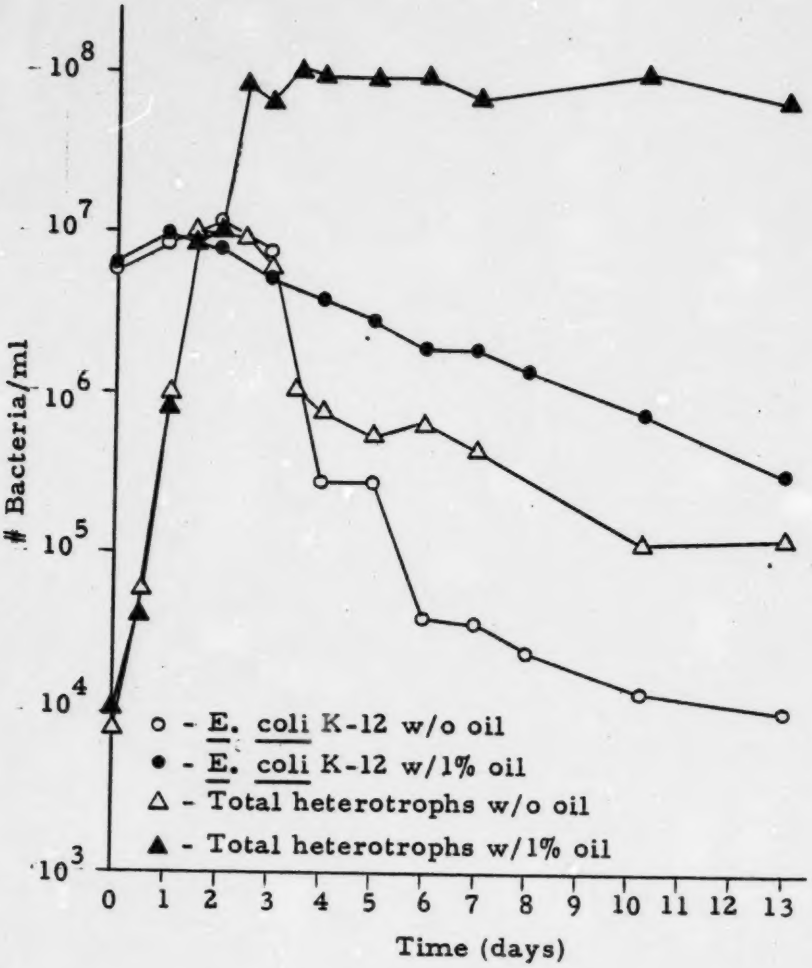

Figure 6. Growth curves of *Escherichia coli* K-12 and naturally-occurring heterotrophic bacteria in salts-supplemented April Chena River water samples with and without 1% Prudhoe Bay crude oil at 20 C.

than in the flask without oil, suggesting that the oil had an inhibitory effect on the lethal factor in raw river water. The heterotrophic bacterial numbers increased in the flask with oil, since they were able to grow on the oil faster than they were killed. The large increase in the number of heterotrophic bacteria in the flask with oil did not accelerate the death of E. coli K-12.

Because the results of the crude oil toxicity tests in raw river water were the opposite of those expected, another study was conducted utilizing crude oil adapted cultures in autoclaved river water to eliminate the lethal factor present in raw river water. The results of this study are shown in Figure 7. The inoculum consisted of 10^4 cells/ml of an adapted culture which had been passed through two successive transfers to fresh Prudhoe Bay crude oil. The initial cell densities of the heterotrophic bacteria from the adapted culture were adjusted to match the cell density in raw river water which can be seen by comparing the number of heterotrophic bacteria in the flasks at zero time in Figures 6 and 7.

Figure 7 shows no decline of E. coli K-12 numbers in the flask without oil after two weeks at 20 C. Heterotrophic bacterial numbers were also stable, indicating that the lethal factor present in raw river water was not present in the adapted cultures. The heterotrophic bacteria in the flask had no apparent adverse effects on E. coli K-12. In the flask with oil, the number of E. coli K-12 decreased by one log

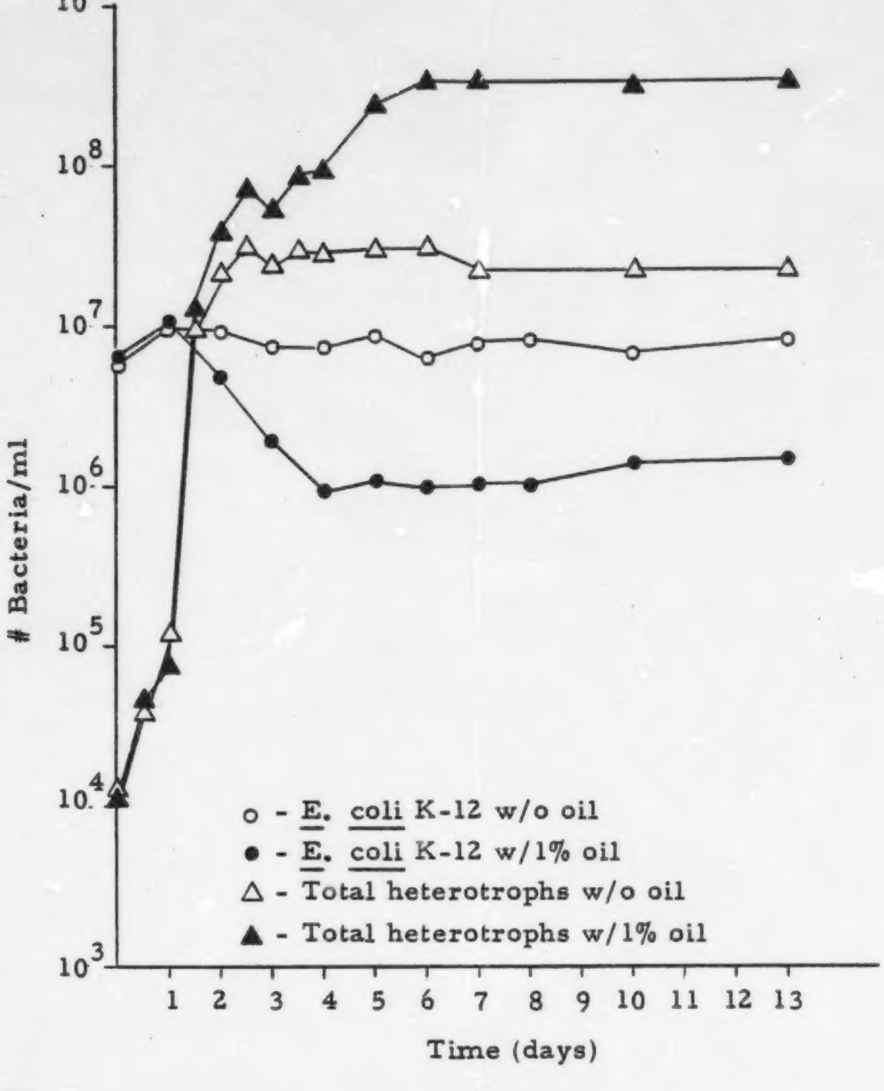

Figure 7. Growth curves of *Escherichia coli* K-12 and crude oil adapted heterotrophic bacteria in autoclaved, salts-supplemented April Chena River water samples with and without 1% Prudhoe Bay crude oil at 20 C.

unit in four days. The decline was greatest during the logarithmic phase of heterotrophic bacterial growth on crude oil. These results indicate that the heterotrophic bacteria formed products during growth on Prudhoe Bay crude oil that were toxic to *E. coli* K-12.

Since the lethal effect of raw river water on *E. coli* K-12 was eliminated by autoclaving and filter sterilizing the water, an attempt was made to characterize the heat-labile biological factor larger than 0.22 micron that was responsible for the decline in *E. coli* K-12 numbers in raw Chena River water. Toxicity studies were performed using March, April and May river water samples and the lethal effect of the water on *E. coli* K-12 was more pronounced with each sample, suggesting that the concentration of lethal factor in the river was increasing as spring breakup approached. Microscopic examination of the contents of flasks in which lethal effects to *E. coli* K-12 were seen revealed the presence of a large number of microorganisms, mostly bacteria and smaller numbers of protozoans. Both ciliated and flagellated protozoans were present in flasks of raw Chena River water without oil, but only flagellated protozoans were present in flasks with oil. Since a decrease in *E. coli* K-12 numbers was seen in flasks with and without oil, it was suspected that a flagellated protozoan was the most likely cause of the decrease in bacterial numbers.

A nutrient agar plate overlaid with soft agar inoculated with *E. coli* K-12 and a small amount of a culture known to be lethal to *E. coli* K-12 was incubated at 30 C and observed daily for clearing in the bacterial lawn. Clearing was detected after one week as patches of irregular size and shape in the lawn of *E. coli* K-12. The cleared patches were examined microscopically under a high dry objective and flagellated protozoans were observed within the cleared zones.

An enrichment culture of the protozoan was made by inoculating a turbid suspension of *E. coli* K-12 in phosphate buffer with a small amount of a culture known to be lethal to *E. coli* K-12. A control suspension of *E. coli* K-12 was also prepared and both suspensions were incubated at room temperature and observed daily for clearing of turbidity. Clearing was seen in the suspension inoculated with the lethal culture within one week, but no clearing was seen in the control suspension. The cleared suspension was examined microscopically in a wet mount preparation and large numbers of a non-pigmented, biflagellated protozoan with a round to oval body were seen among the bacteria singly and in aggregates. No ciliates or other kinds of flagellates were seen. Rod-shaped, refractile forms within the bodies of the protozoans gave evidence that the bacteria were being actively ingested by the protozoans.

The protozoan was keyed to the Order Protomonadida in the Subclass Zoomastigia. The characteristics of the protozoans in this Order are that they are free-living, with two posterior flagella, without chromatophores, with holozoic or saprozoic nutrition and with colonial tendencies (204).

Mutagenicity Testing

The results of mutagenicity testing of whole and fractionated crude oil are presented below.

Mutagenic Activity of Whole Crude Oil

The results of mutagenicity testing of one per cent Black Hollow, Colorado crude oil incubated in artificial salts medium with crude oil adapted cultures are shown in Table 11. Mutagenic activity was detected in test oil extracts after one week at 25 C by Salmonella tester strains TA 1537, TA 1538 and TA 98. Histidine revertant ratios greater than 2.0 were considered positive for mutagenic activity. The histidine revertant ratios shown in Table 11 represent total, microbiologically-mediated and non-biologically-mediated increases in mutagenic activity in the Black Hollow crude oil. No significant increases in mutagenic activity were detected in control oil extracts.

The three Salmonella tester strains which responded positively to the test oil extracts were all sensitive to frameshift mutagens, indicating that base-pair substitution mutagens were not formed in

Table 11. Average number of histidine revertants on triplicate mutagenicity test and control plates and histidine revertant ratios for total, microbiologically-mediated and non-biologically-mediated mutagenic activity in test and control oil extracts of 1% Black Hollow, Colorado crude oil incubated for one week at 25 C with and without a crude oil adapted microbial culture, respectively.

Extract	Tester strain				
	TA 100	TA 1535	TA 1537	TA 1538	TA 98
Solvent[a] Control	77	26	6	12	17
Control[b] Oil	72	29	10	17	20
Test Oil[c]	56	46	24	98	92
Test Oil[d] / Solvent Con.	0.7	1.8	4.0	8.1	5.4
Test Oil[e] / Con. Oil	0.8	1.6	2.4	5.8	4.6
Con. Oil[f] / Solvent Con.	0.9	1.1	1.7	1.4	1.2

[a] 0.1 ml dimethyl sulfoxide added to solvent control plates.

[b] Autoclaved river water in control oil flask uninoculated.

[c] Autoclaved river water in test oil flask inoculated with crude oil adapted microbial culture 1:100 (v/v).

[d] Histidine revertant ratio for total mutagenic activity.

[e] Histidine revertant ratio for microbiologically-mediated mutagenic activity.

[f] Histidine revertant ratio for non-biologically-mediated mutagenic activity.

Note: Histidine revertant ratios of 2.0 or more indicate a significant increase in mutagenic activity.

the Black Hollow crude oil by microorganisms. Extracts of fresh Black Hollow crude oil were not mutagenic to the Salmonella tester strains, showing that the level of mutagens in the oil prior to microbial degradation was too low to be detected by the Ames test. The increases in mutagenic activity shown in Table 11 represent the maximum positive responses detected in Black Hollow crude oil. Weaker positive responses were detected by the same Salmonella tester strains when 0.5 per cent crude oil was tested under the same conditions.

Figure 8 shows the results of a mutagenicity test that was positive for microbiologically-mediated mutagenic activity. The histidine revertant ratios obtained for this test from triplicate test and control plates are presented in Table 12. The discrete colonies on the plates are the histidine revertants and the hazy background lawns contain the unreverted Salmonella tester strains. All of the tester strains except TA 1535 showed a significant increase in histidine revertants in the presence of the microbiologically-degraded oil.

Tables 12 and 13 are a compilation of mutagenicity test results obtained from extracts of Prudhoe Bay crude oil after incubation with salts-supplemented Chena River water samples for various times at 20 and 4 C, respectively. Prudhoe Bay crude oil was routinely tested for mutagenic activity after incubation at 20 C, as the most consistent

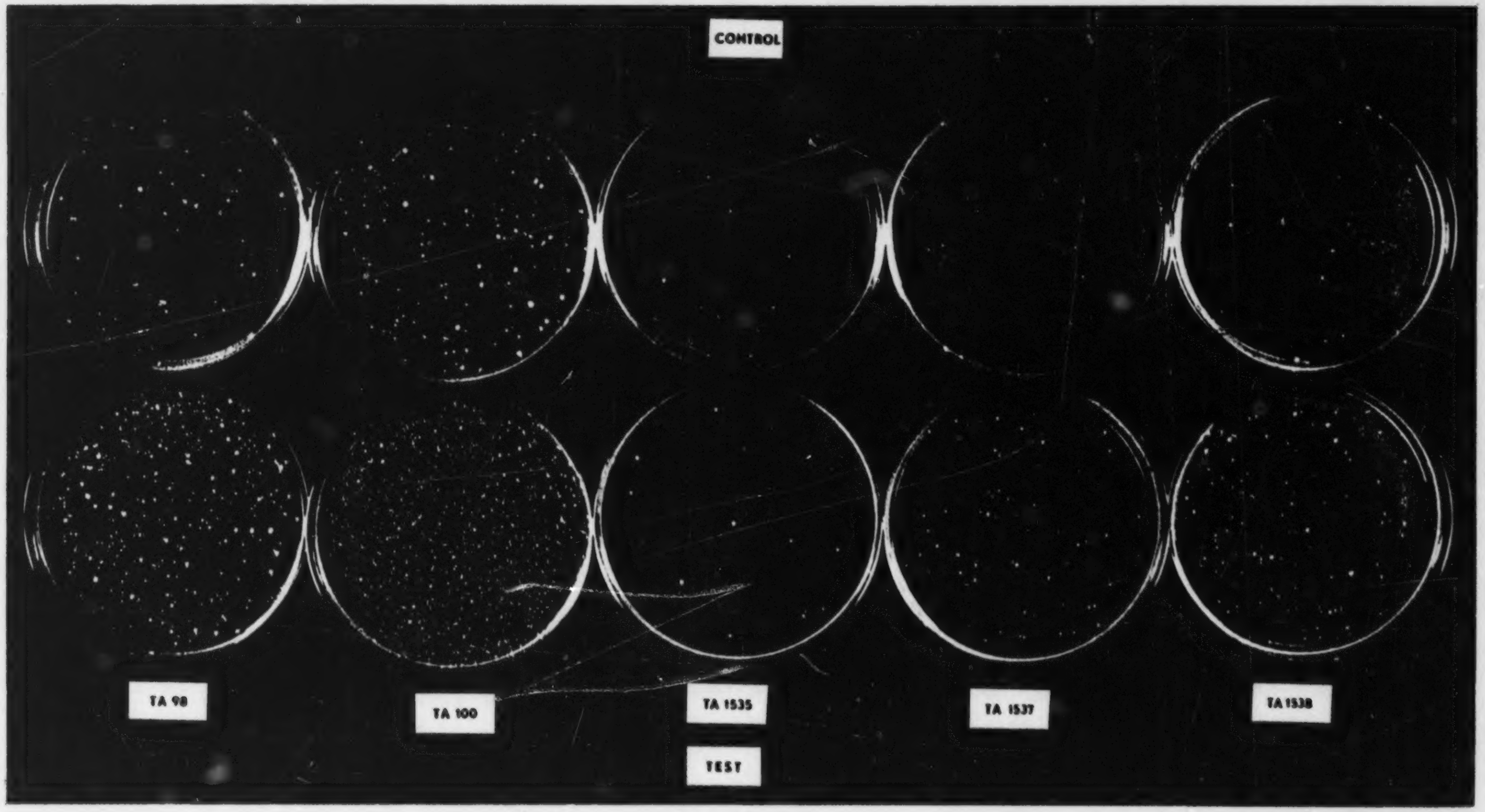
CONTROL
TA 98
TA 100
TA 1535
TA 1537
TA 1538
TEST

Figure 8. Histidine revertant responses of Salmonella tester strains to extracts of 1% Prudhoe Bay crude oil incubated with raw (test) and sterile (control), salts-supplemented October Chena River water samples for one week at 20 C.

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Table 12. Histidine revertant ratios for total/microbiologically-mediated mutagenic activity in test oil extracts of 1% Prudhoe Bay crude oil incubated for various time intervals at 20 C with salts-supplemented Chena River water samples.

Sample Date 1977	Extracted	Tester strain				
		TA 100	TA 1535	TA 1537	TA 1538	TA 98
1/31	2	2.7/1.1*	1.1/1.6	1.9/1.2	2.1/1.2	1.5/1.0
	5	2.8/1.3*	1.0/0.9	2.1/1.8	2.1/1.4	2.2/1.0
3/1	2	1.4/2.0	0.7/1.0	1.5/0.9	1.7/0.9	1.7/0.8
	4	2.1/1.8	1.3/1.3	2.7/1.2*	3.6/1.2*	2.4/1.5
	6	1.3/1.8	1.2/1.3	1.6/0.8	2.4/1.3	1.8/1.0
4/1	4	3.6/1.9	0.8/0.9	1.5/1.2	1.5/1.0	2.5/1.6
5/6	1	3.9/4.7	0.9/1.3	4.4/4.4	7.3/5.5	6.2/5.7
	2	5.3/4.0	1.9/0.8	4.7/3.7	6.1/5.5	5.0/6.0
	4	2.3/2.0	1.3/1.4	2.3/2.6	3.1/2.9	3.0/2.2
6/20	1	4.8/5.2	0.8/0.9	4.0/4.6	9.0/4.8	7.0/5.9
	2	1.2/1.3	0.9/1.0	2.1/1.3	1.4/1.8	1.6/1.0
	3	6.0/4.8	1.4/1.6	4.4/3.3	15.2/6.2*	9.6/5.3
	4	1.3/1.1	1.1/0.9	1.8/1.0	1.8/0.7	1.7/0.9
	5	2.5/2.8	1.1/1.0	2.5/2.1	5.6/5.3	4.3/3.9
7/21	1	1.4/1.3	1.2/1.1	1.7/1.4	3.6/1.6*	1.8/1.3
	2	1.0/0.8	1.1/1.0	0.9/0.8	2.6/1.1*	1.6/1.1
	3	3.6/4.0	1.4/1.2	1.7/1.7	6.7/2.8*	3.6/3.0
	4	2.2/2.0	0.7/0.7	1.3/1.1	2.0/1.3	2.7/1.5

Table 12. (Continued)

Sample Date 1977	Extracted	Tester strain				
		TA 100	TA 1535	TA 1537	TA 1538	TA 98
8/11	1	2.6/2.4	0.7/0.7	2.0/2.9	3.6/3.4	3.6/2.4
	2	2.1/2.3	0.6/1.3	2.3/1.7	3.3/2.7	3.4/2.0
9/12	1	1.9/1.6	0.8/0.9	1.5/1.2	1.7/1.9	2.2/1.3
	2	4.9/4.9	1.2/1.3	3.4/3.8	8.6/5.7	8.7/5.1
	3	4.8/4.4	1.5/1.7	4.0/3.1	8.3/3.5*	8.9/4.0*
	4	4.4/4.0	0.8/1.1	1.3/1.9	3.6/2.2	3.7/2.2
9/16	1	3.9/3.5	0.8/1.0	2.8/1.8	5.4/3.0	7.4/2.6*
	2	3.8/2.9	1.2/1.5	3.1/2.2	6.2/2.5*	6.5/2.4*
	3	6.4/5.3	0.9/1.1	2.7/1.9	6.8/3.2*	7.5/2.9*
	4	7.1/5.9	1.0/0.8	4.5/2.8	9.4/4.3*	7.7/3.8*
10/11	1	6.7/6.1	1.8/1.3	7.7/3.8*	8.1/7.4	8.1/6.2
	2	5.2/5.2	0.8/1.1	2.7/3.4	4.6/5.8	6.0/4.6
	3	2.5/-	1.6/-	2.3/-	5.0/-	2.6/-
11/7	1	2.0/2.1	1.0/1.4	2.0/1.2	1.8/1.8	1.4/1.3
	2	6.4/4.3	1.0/1.2	2.8/1.6	5.9/2.7*	7.5/2.4*
	3	2.6/2.4	0.9/1.0	1.2/1.2	2.0/2.3	2.5/1.8

*Denotes a significant increase in non-biologically-mediated mutagenic activity in control oil extract.

Table 13. Histidine revertant ratios for total/microbiologically-mediated mutagenic activity in test oil extracts of 1% Prudhoe Bay crude oil incubated for various time intervals at 4 C with salts-supplemented Chena River water samples.

Sample Date 1977	Week Extracted	Tester strain				
		TA 100	TA 1535	TA 1537	TA 1538	TA 98
3/1	2	0.9/1.0	1.1/1.6	1.8/1.0	1.9/1.0	2.1/1.0*
	4	1.4/1.2	1.1/1.4	2.2/0.8*	3.2/1.2*	2.4/1.0*
	6	1.7/1.4	1.5/1.0	3.8/1.3*	3.6/1.3*	3.4/1.1*
6/20	1	0.9/0.9	0.5/0.8	0.9/1.0	1.9/1.0	1.2/0.8
	2	0.9/1.0	0.9/1.1	2.0/1.4	1.0/1.2	1.4/0.9
	3	1.6/1.3	1.3/1.6	0.7/0.7	1.3/1.3	1.1/0.8
	4	4.0/4.0	1.3/1.4	4.2/2.3	6.6/4.4	6.1/5.1
	5	2.0/2.2	0.9/1.1	2.0/2.0	4.6/5.1	4.5/3.2
9/12	1	1.0/0.8	0.6/1.2	1.4/0.7	2.3/0.6*	2.0/0.5*
	2	1.2/1.0	1.1/1.4	1.3/1.1	2.8/1.2*	2.5/0.8*
	3	1.5/1.4	1.0/1.2	1.4/2.3	2.2/1.1*	2.6/1.3*
	4	1.7/1.5	1.0/1.0	2.9/3.2	2.7/1.5	3.2/1.6*
9/16	1	1.1/1.1	0.8/0.8	1.2/0.9	1.0/0.8	1.5/0.9
	2	1.1/1.0	0.7/0.5	1.1/1.0	1.7/1.1	2.5/1.5
	3	1.2/1.1	1.2/1.1	1.9/1.4	1.9/1.0	2.1/1.0*
	4	1.8/1.8	0.9/1.5	1.2/1.3	3.0/1.6	2.5/1.2*

*Denotes a significant increase in non-biologically-mediated mutagenic activity in control oil extract.

results were obtained at this temperature and the tests could be performed in less time than at 4 C.

In contrast to Black Hollow crude oil, extracts of Prudhoe Bay crude oil from control flasks were occasionally positive for mutagenic activity. Fresh Prudhoe Bay crude oil was never positive for mutagenic activity and control oils became positive only after incubation with shaking. Increases in the mutagenic activity of control oil extracts were indicative of non-biological activation of the oil to a mutagenic state. Control oils containing non-biologically-mediated mutagenic activity are indicated in Tables 12 and 13.

Tables 12 and 13 show that, with one exception in January, only the Salmonella tester strains sensitive to frameshift mutagens (TA 1537, TA 1538, TA 98) responded positively to non-biologically-activated control extracts of Prudhoe Bay crude oil. The apparent exception in the January water samples was caused by contamination of the control oil flasks by bacteria. Detection of base-pair substitution mutagens by Salmonella tester strain TA 100 occurred only after microbiological degradation of the Prudhoe Bay crude oil had taken place. The appearance of base-pair substitution mutagens in Prudhoe Bay crude oil was indicative of microbiologically-mediated activation of the crude oil to a mutagenic state, since the presence of microorganisms was necessary for their formation. Tables 12 and 13 also show that Salmonella tester strain TA 1535 did not detect mutagens in Prudhoe Bay crude oil under any of the conditions tested.

A distinction between non-biological and microbiological mediation of mutagenic activity in Prudhoe Bay crude was possible, since base-pair substitution mutagens were not present in non-biologically activated crude oil and the level of frameshift mutagens was generally higher in microbiologically activated crude oil than in control oil. The mutagenic activity in control oil extracts can be determined in Tables 12 and 13 by dividing the numbers shown. Although no base-pair substitution mutagens were detected in Black Hollow crude oil, microbiological degradation was necessary for the production of frameshift mutagens in this oil. The results of the above studies show that frameshift mutagens were formed in crude oil by non-biological or microbiological processes, but that base-pair substitution mutagens were formed only by microbiological processes.

The data in Table 14, obtained from Table 12, shows the highest level of microbiologically-mediated mutagenic activity detected in Prudhoe Bay crude oil after exposure to Chena River water samples at various times of the year. No significant increases in the mutagenic activity of Prudhoe Bay crude oil were seen until a May river water sample was tested. The jump in the mutagenic activity of Prudhoe Bay crude oil extracts incubated with May water samples coincided with the beginning of ice breakup in the Chena River. After

Table 14. Effect of seasonal variations in the Chena River on the incubation time and Salmoneila tester strain required to detect the highest level of microbiologically-mediated mutagenic activity in test oil extracts of 1% Prudhoe Bay crude oil incubated at 20 C with salts-supplemented Chena River water samples.

Sample date	Week mutagenic activity detected	Week highest activity detected	Tester strain detecting highest activity	Highest revertant ratio test:control
1/31/77	-	5	TA 1537	1.8
3/1/77	-	2	TA 100	2.0
4/1/77	-	4	TA 100	1.9
5/6/77	1	2	TA 98	6.0
6/20/77	1	3	TA 1538	6.2
7/21/77	3	3	TA 100	4.0
8/11/77	1	1	TA 1538	3.4
9/12/77	2	2	TA 1538	5.7
9/16/77	1	4	TA 100	5.9
10/11/77	1	1	TA 1538	7.4
11/7/77	2	2	TA 100	4.3

breakup, mutagenic activity was usually detected in Prudhoe Bay crude oil within 1-2 weeks of incubation with Chena River water samples at 20 C. Peak levels of mutagenic activity occurred shortly after initial detection, in most cases. The Salmonella tester strain with the greatest response to the mutagens in the oil fluctuated between TA 100, a base-pair substitution mutant, and TA 1538, a frameshift mutant. Both types of mutagens were apparently produced at nearly identical levels of sensitivity in the Prudhoe Bay crude oil during microbiological degradation and one type of mutagen was not produced in preference to the other. The actual concentration of each type of mutagen was probably different due to differences in the sensitivity of the Salmonella tester strains.

Figure 9 shows the response of the five Salmonella tester strains to Prudhoe Bay crude oil extracts incubated with salts-supplemented Chena River water over time. The level of mutagenic detected in the crude oil at different time intervals followed the general pattern shown. An initial lag was followed by a rapid increase in mutagenic activity and a gradual decline to baseline levels. All of the Salmonella tester strains except TA 1535 responded positively to mutagens in extracts of crude oil after microbial degradation, confirming the results in Tables 12 and 13.

The effects of temperature and nutrients on oil degradation were presented earlier in Figures 4 and 5. The effects of these

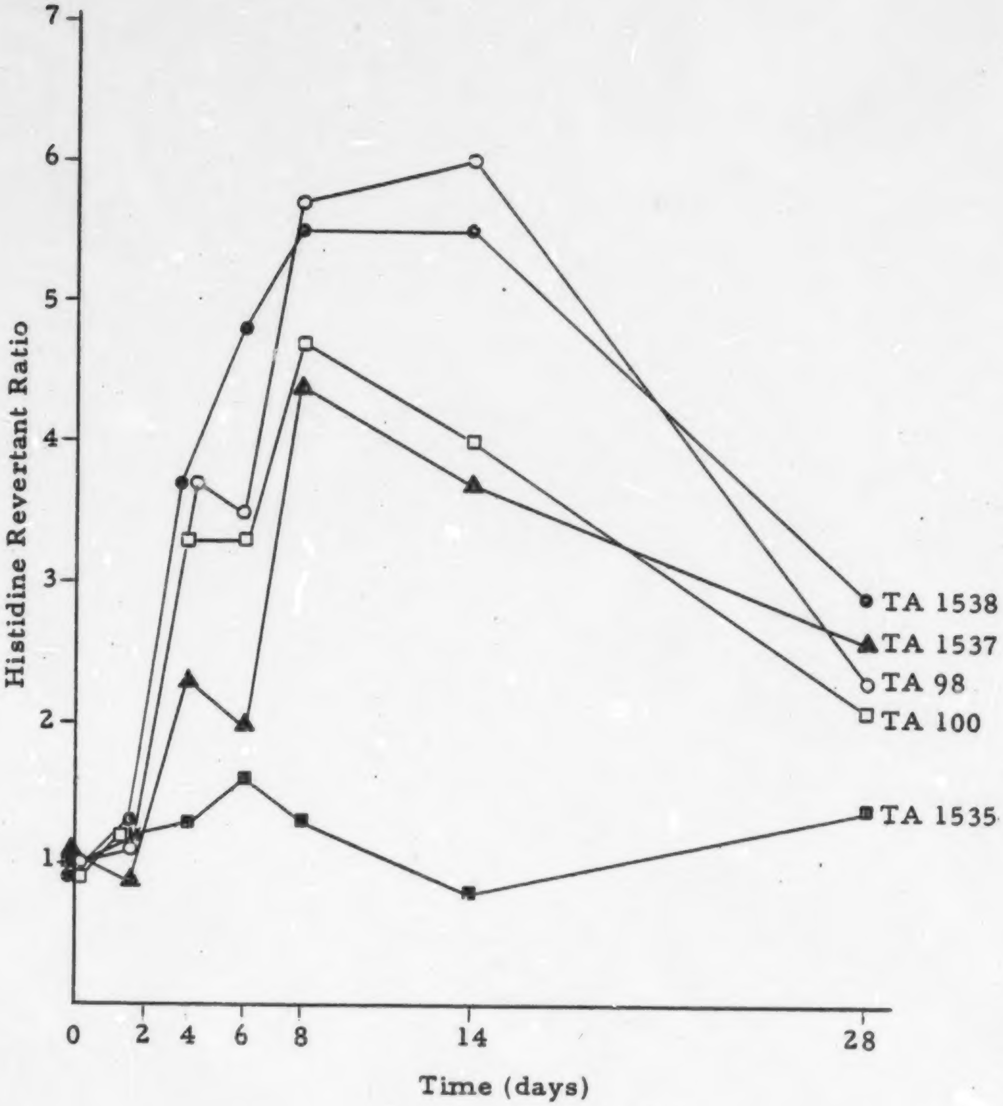

Figure 9. Effect of incubation time on histidine revertant ratios for microbiologically-mediated mutagenic activity detected by each Salmonella tester strain in extracts of 1% Prudhoe Bay crude oil incubated with salts-supplemented May Chena River water samples at 20 C.

same variables on the expression of mutagenic activity in Prudhoe Bay crude oil during microbial degradation are shown in Table 15. There was no significant increase in the microbiologically-mediated mutagenic activity of Prudhoe Bay crude oil at 4 or 20 C after incubation with raw, unsupplemented Chena River water for five weeks. The microbiologically-mediated mutagenic activity of the oil increased at both 4 and 20 C after incubation in salts-supplemented Chena River water. Mutagenic activity was detected in the oil after one week at 20 C, but not until the fourth week at 4 C. The appearance of mutagenic activity in the oil correlates with the times required to reach the stationary phase of heterotrophic bacterial growth and to detect a weight loss in the oil at both temperatures, as shown in Figures 4 and 5. The maximum level of mutagenic activity in the crude oil occurred in the stationary phase of microbial growth on the oil. The peak and decline in mutagenic activity over time can be seen in Table 15.

Irregularities in the expression of mutagenic activity in the Prudhoe Bay crude oil extracts incubated at 20 C can be seen in Table 15. Significant mutagenic activity was detected in 1, 3 and 5 week oil extracts, but not in 2 and 4 week oil extracts. These results could be interpreted as an undulating pattern of mutagenic activity in the oil over time, but this does not agree with the data in Figure 9. The absence of mutagenic activity in oil extracts at weeks

Table 15. Effects of temperature and inorganic nitrogen and phosphorus salts on histidine revertant ratios for microbiologically-mediated mutagenic activity detected by each Salmonella tester strain in test oil extracts of 1% Prudhoe Bay crude oil incubated with May Chena River water samples over time.

Extract	Week Extracted	Tester strain				
		TA 100	TA 1535	TA 1537	TA 1538	TA 98
20 C Test	1	1.1	0.8	1.4	-	1.2
	2	1.1	0.9	1.5	1.5	1.0
	3	1.1	1.5	0.7	1.1	1.0
	4	1.0	1.0	1.0	0.8	0.8
	5	1.1	0.8	1.1	1.0	1.6
20 C Test N&P	1	5.2	0.9	4.6	4.8	5.9
	2	1.3	1.0	1.3	1.8	1.0
	3	4.8	1.6	3.3	6.2	5.3
	4	1.1	0.9	1.0	0.7	0.9
	5	2.8	1.0	2.1	5.3	3.9
4 C Test	1	0.8	0.9	1.0	0.6	0.7
	2	1.0	1.2	1.3	1.0	0.8
	3	0.9	1.4	0.8	1.4	1.0
	4	1.0	0.7	0.6	0.7	0.9
	5	1.0	0.8	0.6	1.0	0.8
4 C Test N&P	1	0.8	0.9	1.0	1.1	0.8
	2	1.0	1.1	1.4	1.3	0.9
	3	1.3	1.7	0.7	1.3	0.8
	4	4.2	1.4	2.3	4.3	5.0
	5	2.2	1.1	2.0	5.0	3.1

N&P - Nitrogen and phosphorus salts added to river water.

2 and 4 was thought to be due to variability in oil degradation among replicate flasks instead. To test this possibility, 10 replicate flasks containing one per cent Prudhoe Bay crude oil and salts-supplemented Chena River water were incubated at 20 C under identical conditions. The oil was extracted from the flasks after two weeks and tested for mutagenic activity. The results of this study are shown in Table 16. Considerable variation can be seen in the amount of microbiologically-mediated mutagenic activity in replicate extracts from no activity in replicate 5 to strong activity in replicates 4, 6 and 7.

The data in Tables 10 and 16 were obtained from the same oil degradation flasks. Some trends can be seen in the appearance of the flasks and the amount of mutagenic activity detected in the oil extracts. Flasks with globular oil emulsions and yellow-orange aqueous phases were the most consistent producers of mutagenic activity throughout the study. Flasks containing crude oil adapted cultures tended to depart from this appearance and generally had lower levels of mutagenic activity than flasks containing unadapted cultures.

Figure 10 shows the effect of crude oil concentration on the expression of microbiologically-mediated mutagenic activity in Prudhoe Bay crude oil. The highest level of mutagenic activity in oil degradation flasks occurred when the initial concentration of Prudhoe Bay crude oil was about one per cent (w/v). Decreases in mutagenic

Table 16. Average number of histidine revertants on mutagenicity test (R1-R10) and control oil plates and histidine revertant ratios for microbiologically-mediated mutagenic activity in extracts from ten replicate oil degradation flasks after incubation of salts-supplemented October Chena River water samples and 1% Prudhoe Bay crude oil for two weeks at 20 C.

Replicate	Tester strain				
	TA 100	TA 1535	TA 1537	TA 1538	TA 98
Control oil	192	27	12	15	52
R1	527	39	25	31	107
	2.7	1.4	2.1	2.1	2.0
R2	530	30	26	32	118
	2.8	1.1	2.2	2.1	2.3
R3	503	25	28	51	130
	2.6	0.9	2.3	3.4	2.5
R4	965	33	49	69	256
	5.0	1.2	4.1	4.6	4.9
R5	348	25	20	23	84
	1.8	0.9	1.7	1.5	1.6
R6	1032	31	41	83	240
	5.4	1.1	3.4	5.5	4.6
R7	700	36	40	62	198
	3.6	1.3	3.3	4.1	3.8
R8	419	26	22	38	110
	2.2	1.0	1.8	2.5	2.1
R9	455	34	24	43	116
	2.4	1.2	2.0	2.9	2.2
R10	336	29	22	36	93
	1.8	1.1	1.8	2.4	1.8

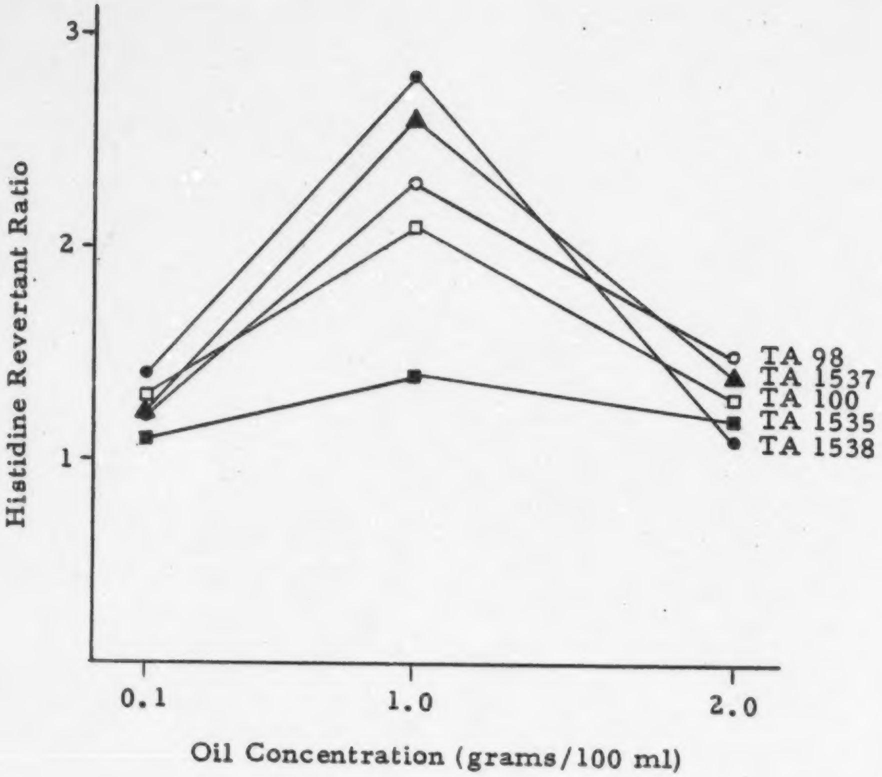

Figure 10. Effect of initial crude oil concentration on histidine revertant ratios for microbiologically-mediated mutagenic activity detected by each Salmonella tester strain in extracts of Prudhoe Bay crude oil incubated with salts-supplemented May Chena River water samples for four weeks at 20 C.

activity were seen at crude oil concentrations above and below this level. This effect was confirmed in several trials and occurred with all of the Salmonella tester strains except TA 1535.

Oil extracts from nine replicate oil degradation flasks incubated for three weeks at 20 C were pooled and tested for mutagenic activity at increasing concentrations to establish dose response curves for the Salmonella tester strains. The results of this study are shown in Figure 11. The oil concentrations were increased by 1.5X increments over the concentration range tested. The correlation coefficient (R) of each set of plotted points was determined by linear regression analysis to check the linearity of response of the Salmonella tester strains to the mutagens in the oil. Linear dose responses were obtained for all of the tester strains except TA 1535.

The dose response data was replotted in Figure 12 to determine the minimum concentration of oil extract needed to yield a positive mutagenicity test with each Salmonella tester strain. Tester strain TA 1538 was most sensitive to the mutagens in the oil extract, giving a positive mutagenic response with 0.1 gram of extract. Tester strains TA 1537, TA 100 and TA 98 had nearly the same sensitivity to the mutagens in the oil extract, giving positive responses with 0.33 gram of extract. From 0.5-0.75 gram of oil was generally recovered from oil degradation flasks initially containing one gram of fresh Prudhoe Bay crude oil. Figure 12 shows that mutagenic

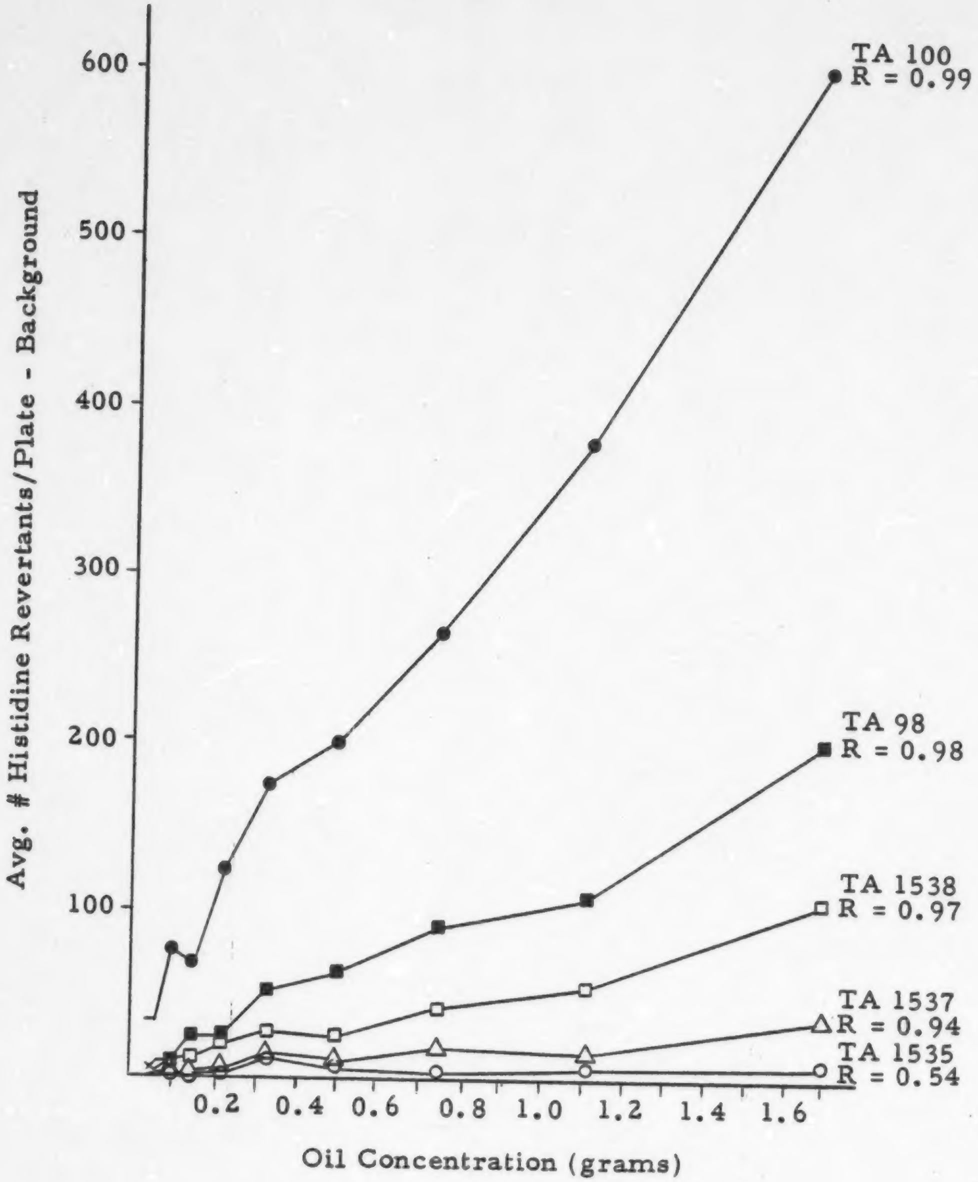

Figure 11. Dose response curves of Salmonella tester strains to nine pooled extracts of 1% Prudhoe Bay crude oil incubated with salts-supplemented October Chena River water samples for three weeks at 20 C as the oil concentration is increased by 1.5 X increments and linear correlation coefficients (R) for each curve.

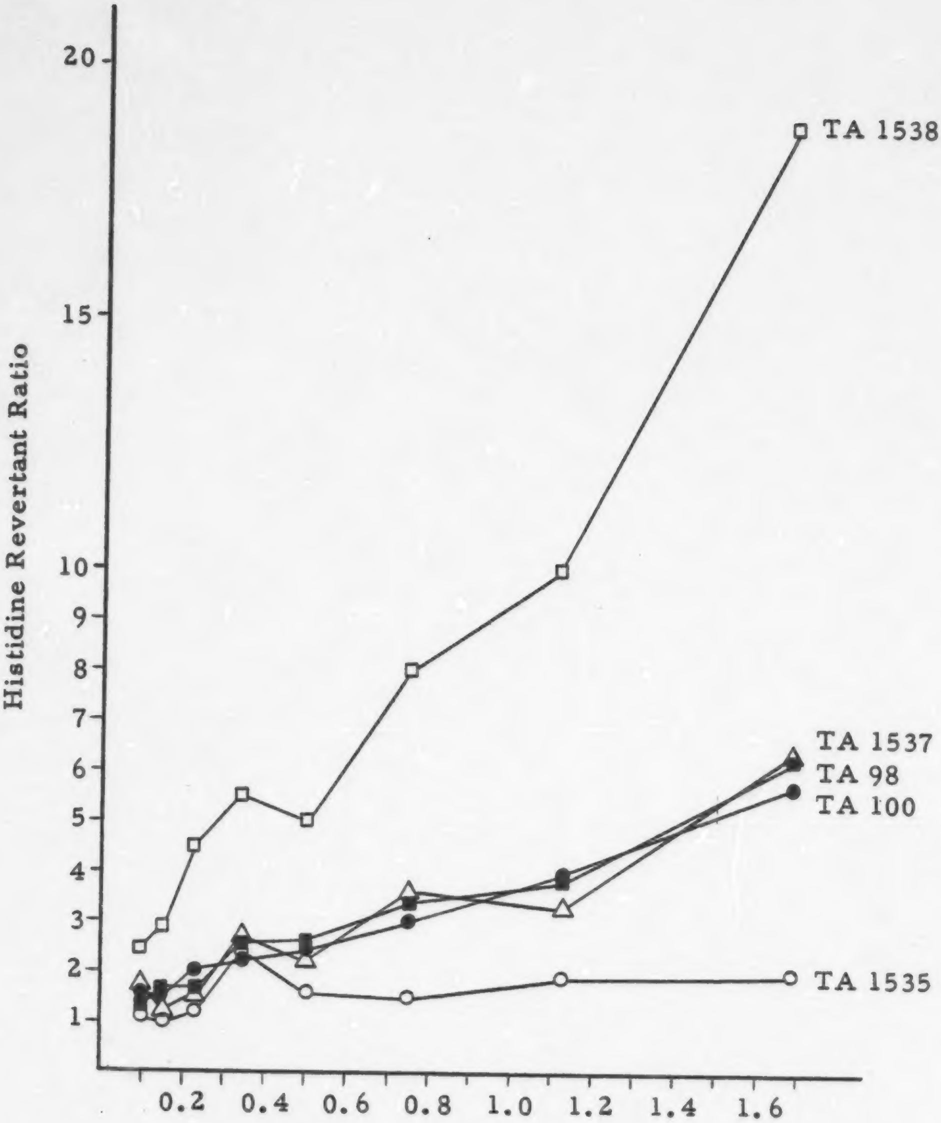

Figure 12. Histidine revertant ratios for total mutagenic activity detected by Salmonella tester strains in nine pooled extracts of 1% Prudhoe Bay crude oil incubated with salts-supplemented October Chena River water samples for three weeks at 20 C as the oil concentration is increased by 1.5 X increments.

activity was detected by the Ames test within this weight range of oil extract.

Figures 13-17 show the relative amount of mutagenic activity contributed by non-biological and microbiological activation processes in Prudhoe Bay crude oil incubated with each salts-supplemented Chena River water sample at 20 C. The data in these Figures were obtained from Table 12 and represent the highest levels of mutagenic activity detected by the Salmonella tester strains in oil extracts from a given water sample. The abscissas were plotted according to the time the water samples were taken to show the effect of seasonal changes in the Chena River on the expression of mutagenic activity in Prudhoe Bay crude oil. TA 1535 was the only Salmonella tester strain which did not give a positive response to the mutagens in Prudhoe Bay crude oil at any time. Tester strain TA 1537 appeared to be less responsive to the mutagens in the oil extracts than tester strains TA 100, TA 1538 and TA 98. The positive mutagenic response of tester strain TA 100 to a control oil extract in January was inconsistent with subsequent observations and did not occur in any other tests.

An attempt was made to correlate seasonal changes in the Chena River with the increases and decreases in microbiologically-mediated mutagenic activity in Prudhoe Bay crude oil incubated with river water samples at different times of the year. Table 17 lists

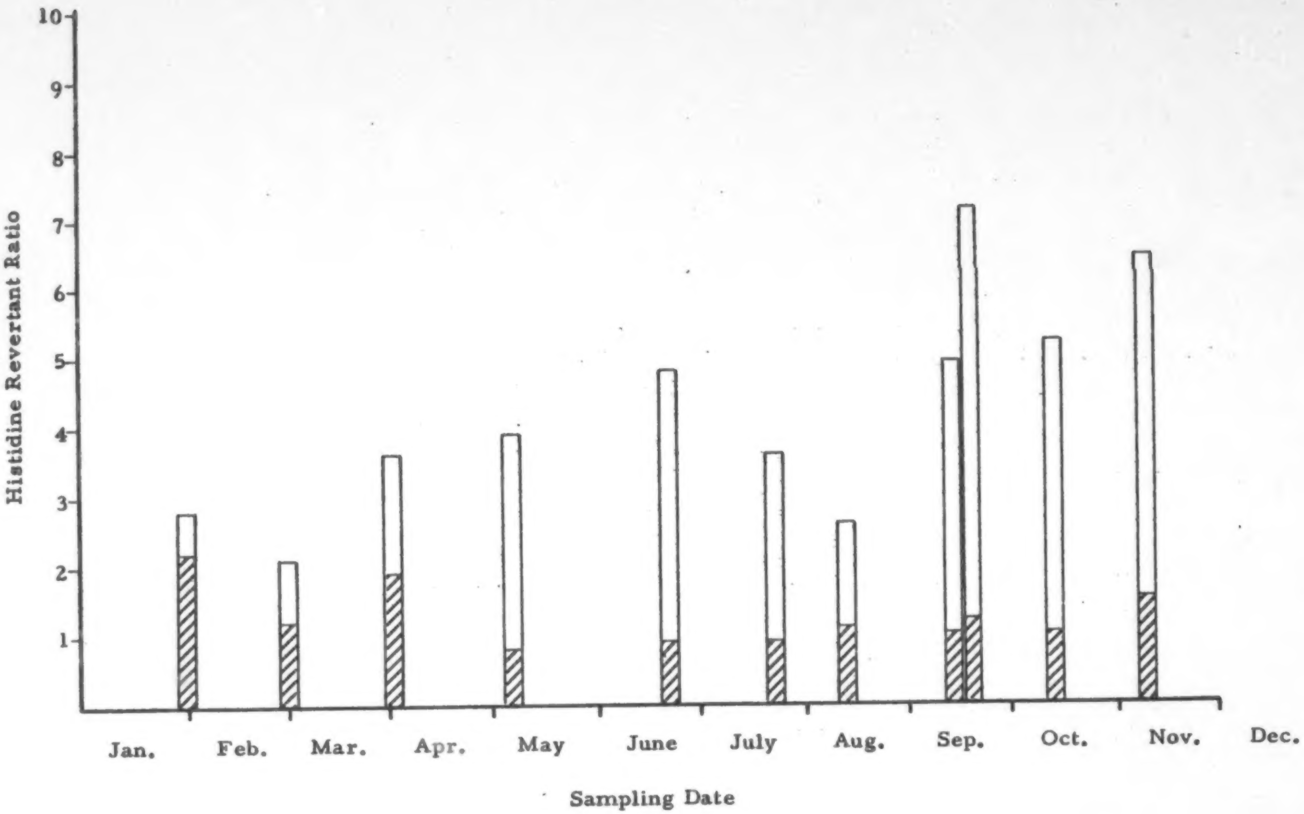

Figure 13. Effect of seasonal changes in the Chena River on histidine revertant ratios for total (total bar height), non-biologically-mediated (cross-hatched bar height) and microbiologically-mediated (open bar height) mutagenic activity detected by Salmonella tester strain TA 100 in extracts of 1% Prudhoe Bay crude oil with the highest levels of mutagenic activity after incubation with each salts-supplemented river water sample at 20 C.

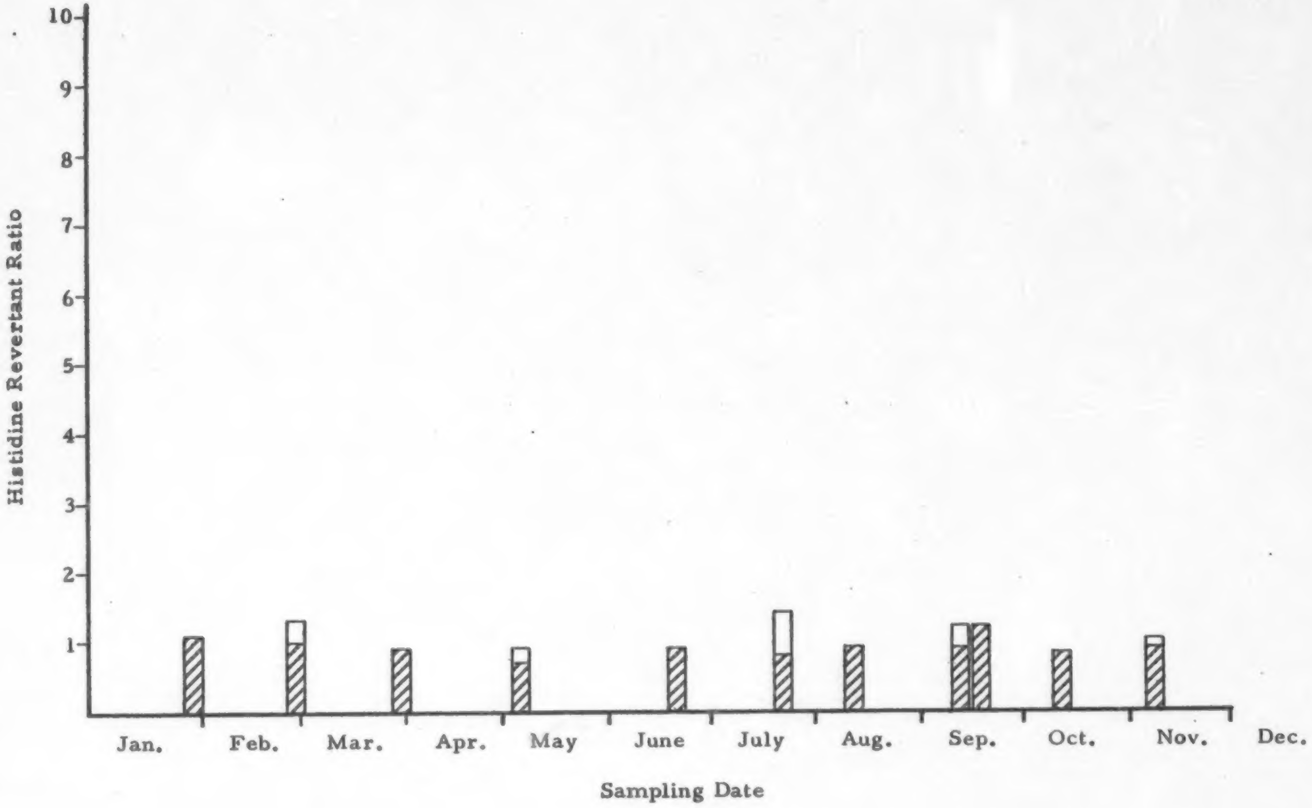

Figure 14. Effect of seasonal changes in the Chena River on histidine revertant ratios for total (total bar height), non-biologically-mediated (cross-hatched bar height) and microbiologically-mediated (open bar height) mutagenic activity detected by Salmonella tester strain TA 1535 in extracts of 1% Prudhoe Bay crude oil with the highest levels of mutagenic activity after incubation with each salts-supplemented river water sample at 20 C.

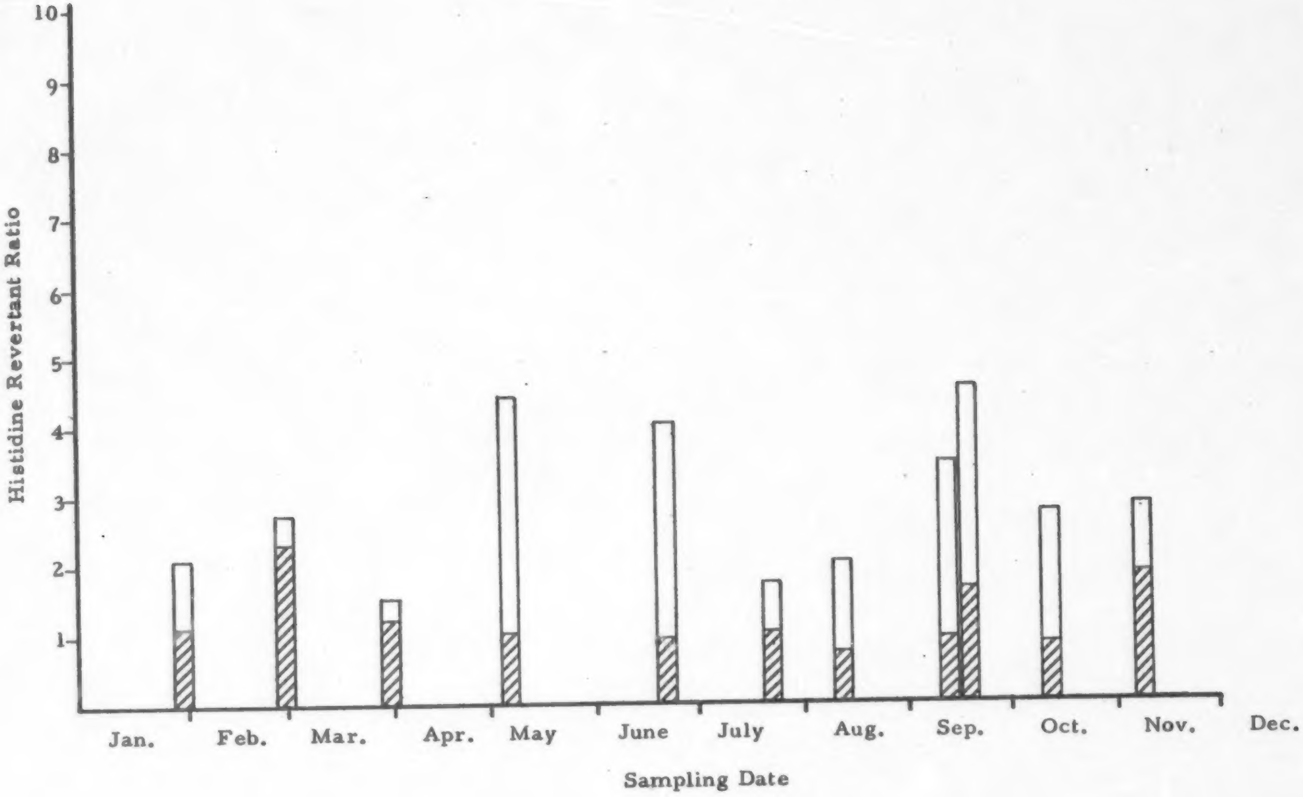

Figure 15. Effect of seasonal changes in the Chena River on histidine revertant ratios for total (total bar height), non-biologically-mediated (cross-hatched bar height) and microbiologically-mediated (open bar height) mutagenic activity detected by Salmonella tester strain TA 1537 in extracts of 1% Prudhoe Bay crude oil with the highest levels of mutagenic activity after incubation with each salts-supplemented river water sample at 20 C.

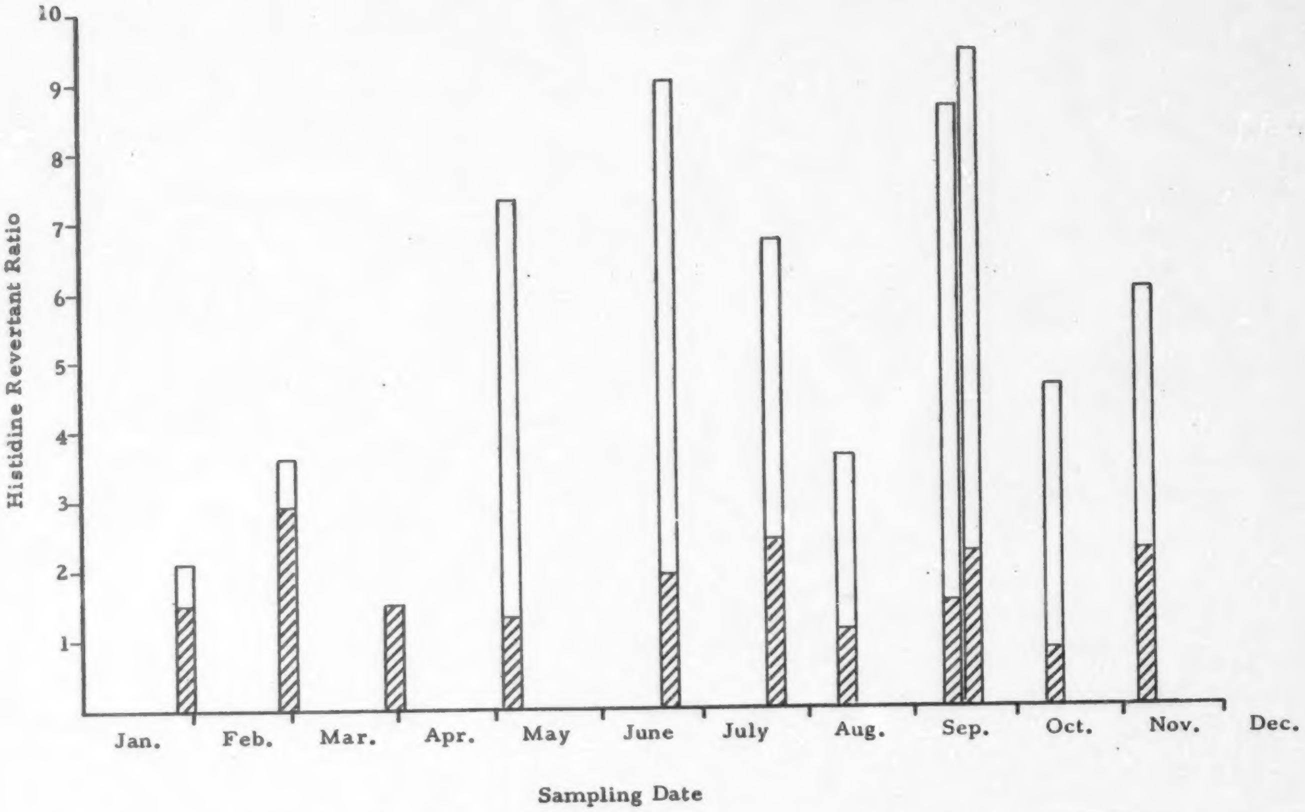

Figure 16. Effect of seasonal changes in the Chena River on histidine revertant ratios for total (total bar height), non-biologically-mediated (cross-hatched bar height) and microbiologically-mediated (open bar height) mutagenic activity detected by *Salmonella* tester strain TA 1538 in extracts of 1% Prudhoe Bay crude oil with the highest levels of mutagenic activity after incubation with each salts-supplemented river water sample at 20 C.

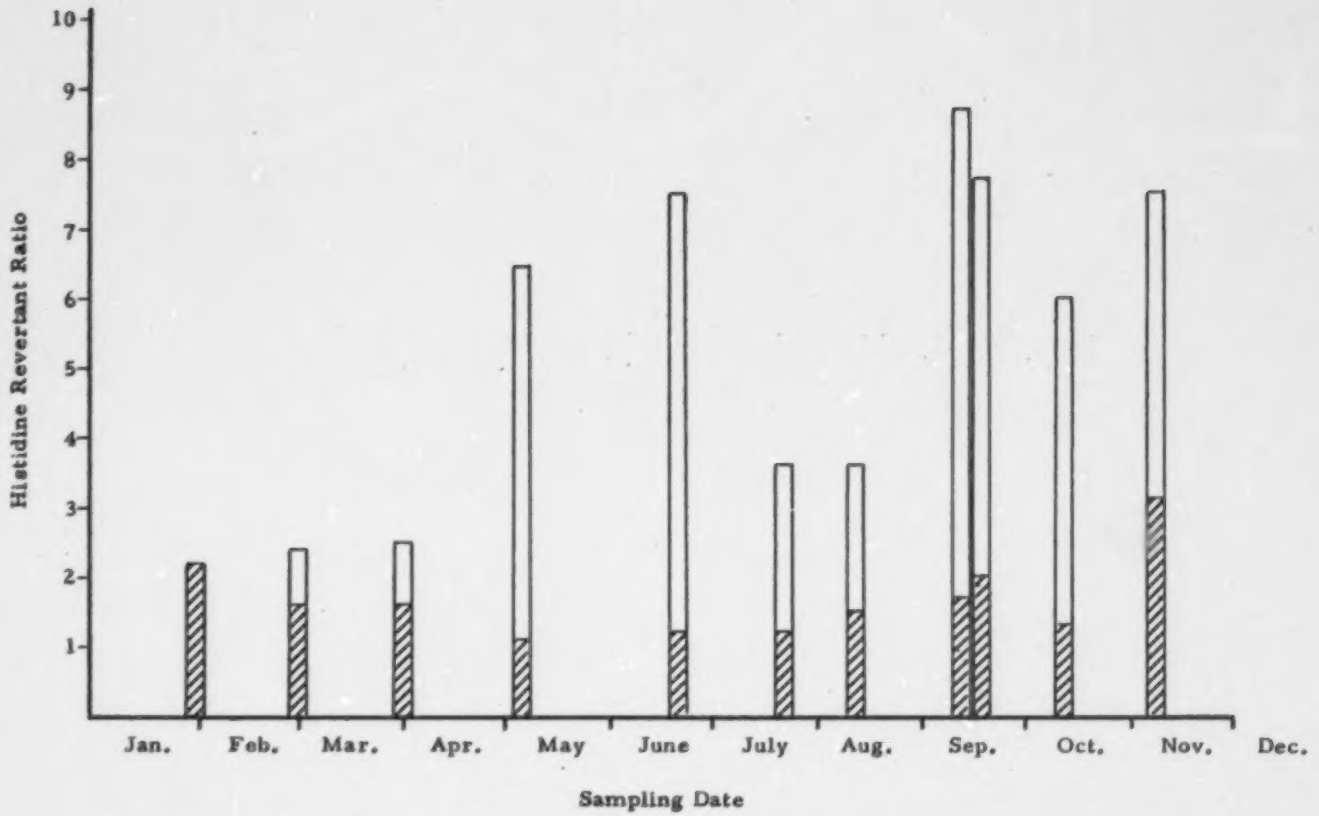

Figure 17. Effect of seasonal changes in the Chena River on histidine revertant ratios for total (total bar height), non-biologically-mediated (cross-hatched bar height) and microbiologically-mediated (open bar height) mutagenic activity detected by Salmonella tester strain TA 98 in extracts of 1% Prudhoe Bay crude oil with the highest levels of mutagenic activity after incubation with each salts-supplemented river water sample at 20 C.

Table 17. Discharge measurements, temperature and heterotrophic bacterial numbers in the Chena River at the time of sampling.

Sample date	Heterotroph count # bact./ml	Temperature C	Discharge* ft^3/sec
1/31/77	3.0×10^3	0.2	160
3/1/77	1.5×10^4	0.2	160
4/1/77	1.4×10^4	0.2	190
5/6/77	1.6×10^5	0.5	3900
6/20/77	5.1×10^4	10.0	4050
7/21/77	-	13.5	1370
8/11/77	3.2×10^5	15.5	1140
9/12/77	3.0×10^5	8.9	1060
9/16/77	1.6×10^5	8.0	1280
10/11/77	8.2×10^3	2.4	2010
11/7/77	3.2×10^3	0.0	670

*Discharge measurements provided by U. S. Geological Survey, Fairbanks, Alaska.

the parameters measured in Chena River water samples from the C-100 station at the time of sampling. Heterotrophic bacterial numbers and temperature reached maximum levels in July and August which corresponded to a decrease in microbiologically-mediated mutagenic activity in Prudhoe Bay crude oil, as shown in Figures 13-17. The daily discharge of the Chena River at the C-100 station in 1977 is shown in Figure 18. Three major increases in the discharge of the river were recorded in May, June and September. The increased discharge in May occurred at the time of ice breakup in the river and was caused by runoff from snow and ice melt. The increased discharge in June resulted from a combination of rain and snow melt. The increase in September was due to fall rains. The peaks in the discharge measurements of the Chena River correspond to peaks in the mutagenic activity of crude oil incubated with river water samples taken at these times. Water runoff from soil appeared to be a major factor in determining the ability of the microorganisms in the river water to increase the mutagenic activity of crude oil.

Mutagenic Activity of Crude Oil Fractions

Prudhoe Bay crude oil was fractionated into various components by column chromatography for mutagenicity testing. Table 18 shows a typical analysis of the fractions present in the oil based on the fractionation of one gram of fresh Prudhoe Bay crude oil. The volatiles, insoluble asphaltenes and insoluble NSO's were not tested

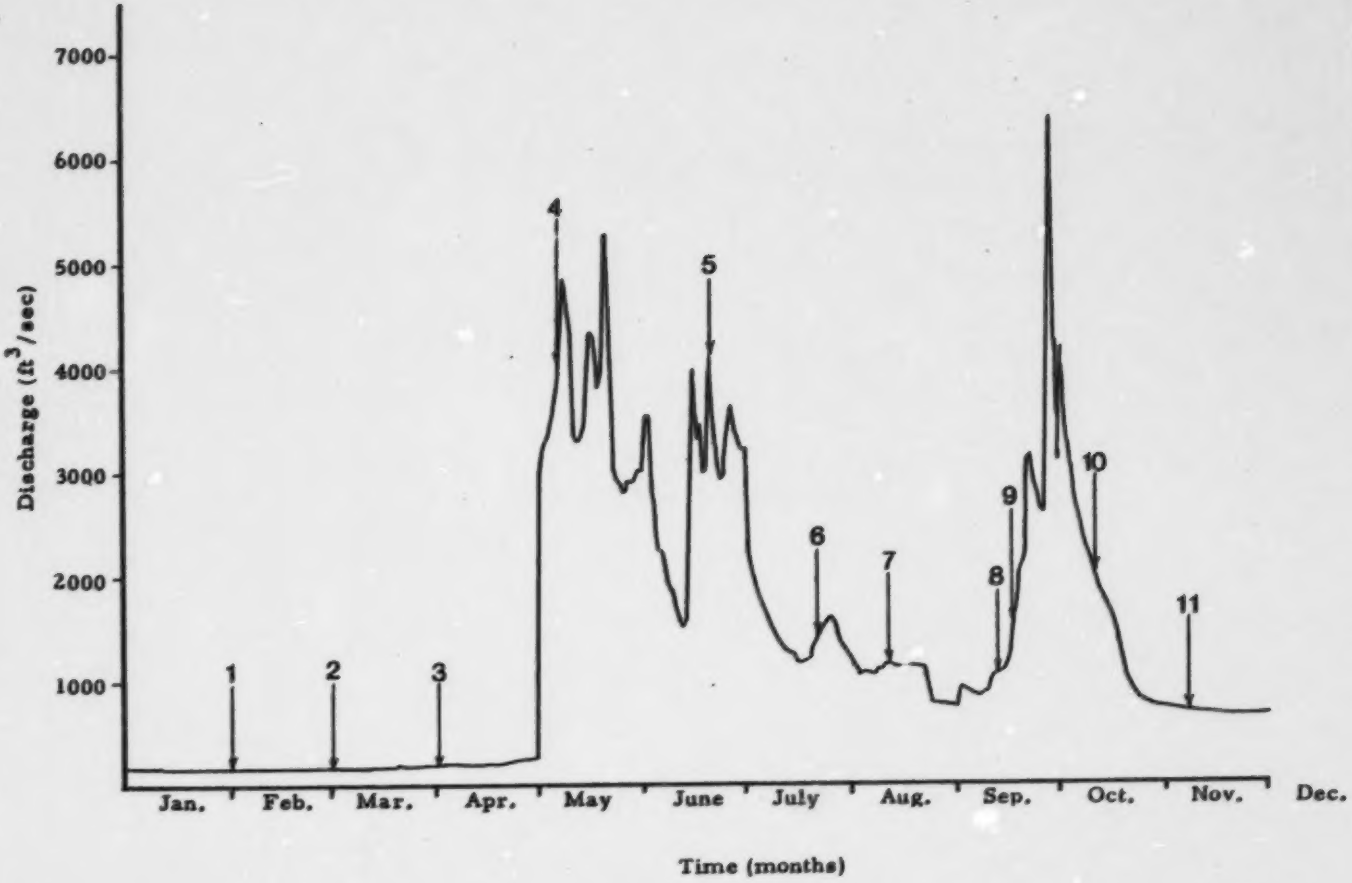

Figure 18. Daily discharge measurements for the Chena River in 1977 and discharge at the time of sampling. Data provided by the U. S. Geological Survey, Fairbanks, Alaska.

Table 18. Analysis of Prudhoe Bay crude oil by column chromatography.

Column	Fraction	Weight* (g)	%
	Whole crude oil	1.0050	100.0
	Volatiles	0.1657	16.5
	Topped oil	0.8394	83.5
HyFlo SuperCel	Deasphaltened oil	0.7798	77.6
	Benzene soluble asphaltenes	0.0403	4.0
	Insoluble asphaltenes	0.0193	1.9
		0.8394	83.5
Silica gel-alumina	Saturates	0.3831	38.1
	Aromatics	0.2748	27.3
	Soluble NSO's	0.0879	8.7
	Insoluble NSO's	0.0340	3.4
		0.7798	77.5

*Average of three samples.

for mutagenic activity, as they were lost in the fractionation procedure. These fractions comprised 21.8 per cent of the whole crude oil. Freon^{TM} extraction of fresh Prudhoe Bay crude oil from water resulted in a weight loss of 21.6 per cent as shown in Figure 5, indicating that these fractions were not recovered from oil degradation flasks for mutagenicity testing either.

Saturate, aromatic and NSO fractions of Prudhoe Bay crude oil were incubated in test and control flasks of salts-supplemented Chena River water for one week at 20 C, extracted with Freon^{TM} 113 and tested for mutagenic activity. The results of this study are shown in Table 19. Significant mutagenic was detected only in the aromatic fraction after incubation. The mutagenic activity in the aromatic fraction was detected by Salmonella tester strains TA 1537, TA 1538 and TA 98, indicating the presence of frameshift mutagens only. Since the mutagenic activity was higher in the control than in the test extract of the aromatic fraction, the mutagens were apparently produced by non-biological activation processes. The lower level of mutagens in the test extract indicates that the non-biologically-mediated mutagens in the aromatic fraction were partially destroyed by microbiological degradation.

Table 20 shows the results of a more extensive study of the mutagenic activity of crude oil fractions. A crude oil adapted culture, passed four times through fresh Prudhoe Bay crude oil,

Table 19. Histidine revertant ratios for non-biologically-mediated mutagenic activity in control extracts and microbiologically-mediated mutagenic activity in test extracts of the saturate, aromatic and NSO fractions derived from one gram portions of fresh Prudhoe Bay crude oil and incubated for one week at 20 C with 100 ml samples of salts-supplemented June Chena River water.

	Tester strain				
Extract	TA 100	TA 1535	TA 1537	TA 1538	TA 98
Saturate Control	1.0	1.0	0.9	1.1	1.0
Saturate Test	0.9	1.0	0.9	1.1	0.9
Aromatic Control	1.1	0.8	3.2	7.5	3.4
Aromatic Test	1.2	1.0	1.8	4.2	2.3
NSO Control	1.1	0.9	0.7	1.9	1.0
NSO Test	1.3	0.8	1.3	1.7	1.2

Table 20. Histidine revertant ratios for non-biologically-mediated mutagenic activity in control extracts and microbiologically-mediated mutagenic activity in test extracts of various crude oil fractions derived from one gram portions of fresh Prudhoe Bay crude oil and incubated for one week at 20 C with 100 ml samples of salts-supplemented November Chena River water.

Fraction	Extract	Tester strain TA 100	TA 1535	TA 1537	TA 1538	TA 98
Crude	Con.	0.7	0.6	0.6	1.0	0.6
	Test	2.5	0.7	1.5	1.2	1.9
*Deasphaltened-benzene soluble asphaltenes	Con.	0.9	0.7	1.2	1.1	1.1
	Test	1.2	0.7	1.3	0.7	1.1
Deasphaltened	Con.	0.9	0.7	1.6	1.2	1.1
	Test	1.1	0.7	1.4	1.4	1.2
*Saturate-aromatic-NSO-benzene sol. asph.	Con.	1.0	0.6	1.8	1.6	1.7
	Test	0.8	0.5	1.0	1.2	1.2
*Saturate-aromatic-NSO	Con.	0.9	0.9	1.9	1.6	1.4
	Test	1.2	0.6	1.5	1.8	1.6
*Saturate-aromatic	Con.	1.0	0.5	2.4	2.0	1.8
	Test	0.9	0.5	1.4	1.6	1.5
*Saturate-NSO	Con.	0.9	0.8	0.8	1.4	0.8
	Test	0.8	0.4	1.0	1.0	1.0
*Aromatic-NSO	Con.	1.0	0.7	1.5	1.4	1.4
	Test	0.8	0.3	1.6	1.2	1.0
Saturate	Con.	0.5	0.6	0.5	0.6	0.4
	Test	0.7	0.6	0.9	0.6	0.8
Aromatic	Con.	0.6	0.7	3.6	3.3	1.8
	Test	0.8	0.3	1.8	1.8	1.5
NSO	Con.	0.9	0.5	0.2	0.9	0.8
	Test	0.9	0.4	0.8	0.9	1.0
Benzene soluble asphaltenes	Con.	0.5	0.6	0.4	0.5	0.4
	Test	0.7	0.4	0.9	0.8	1.0

*Denotes back-addition of components after fractionation.

Note: test flasks inoculated with crude oil adapted culture.

was inoculated into sterile, salts-supplemented Chena River water for this study. Crude oil in various stages of fractionation was tested in this manner to determine if some fractions of the oil masked the expression of mutagenic activity in other fractions. Significant mutagenic activity was detected only in control flasks containing aromatic and saturate-aromatic fractions by Salmonella tester strains TA 1537, TA 1538 and TA 98, confirming the results in Table 19 that only frameshift mutagens were produced in the aromatic fraction of Prudhoe Bay crude oil by non-biological processes and that microbiological degradation destroyed the mutagenic activity in fractionated crude oil. The saturate fraction partially masked the non-biologically-mediated expression of mutagens in the aromatic fraction of Prudhoe Bay crude oil and the NSO fraction completely masked their expression in control extracts of saturate-aromatic and aromatic-NSO fractions, respectively.

Figure 20 shows that Salmonella tester strain TA 100 detected mutagenic activity in the test oil extract of unfractionated Prudhoe Bay crude oil, indicating the presence of microbiologically-mediated mutagenic activity in this extract. Since test oil extracts of fractionated crude oil were not mutagenic in the Ames test, it appears that whole crude oil was necessary for the microbiologically-mediated expression of mutagens.

Data Analysis

The average number of histidine revertants on 29 sets of strain and solvent controls, plated in triplicate for each Salmonella tester strain (87 individual plates/control/tester strain), were statistically analyzed as shown in Table 21. Each set of strain and solvent controls was included with a series of test and control oil extracts being tested for mutagenic activity by the Ames test.

The standard deviations of the average number of histidine revertants on the strain and solvent control plates were calculated to determine if a doubling of the mean number of histidine revertants was indicative of significant mutagenic activity in crude oil extracts. At a 99.5 per cent confidence interval, which is approximately equal to three standard deviations from the mean, the number of histidine revertants at the upper confidence limit was less than or equal to twice the mean for all Salmonella tester strain controls except the solvent control for tester strain TA 1535. As the standard deviation is based on a normal distribution about the mean, only 0.25 per cent (or 1 in 400) of the control plates run in this study would be expected to exceed twice the mean number of histidine revertants. The solvent control plates for Salmonella tester strain TA 1535 had a slightly higher probability of having more than twice the mean number of histidine revertants, but this was not a problem, since this tester strain did not respond positively to the mutagens in crude oil

Table 21. Statistical analysis of the average number of histidine revertants on 29 sets of strain and solvent controls plated in triplicate for each Salmonella tester strain.

Statistical parameter	Tester strain									
	TA 100		TA 1535		TA 1537		TA 1538		TA 98	
	Strain	Solvent	Strain	Solvent	Strain	Solvent	Strain	Solvent	Strain	Solvent
Mean $\overline{x}$	181	161	21	22	10	10	12	13	39	34
Standard deviation $s_{\overline{x}}$	42.5	34.0	6.9	7.8	2.6	2.8	3.4	3.1	9.4	6.0
Confidence interval $\pm t_{0.005}s_{\overline{x}}$	±129	±103	±21	±24	±8	±8	±10	±12	±29	±18
Upper confidence limit $\overline{x} + t_{0.005}s_{\overline{x}}$	310	264	42	46	18	18	22	25	68	52
$2\overline{x}$	362	322	42	44	20	20	24	26	78	68
Paired t test $t_{0.05}$	3.8		-1.0		-1.3		-1.0		3.7	
H_o: $\overline{x}_{strain} = \overline{x}_{solvent}$	Reject		Accept		Accept		Accept		Reject	

For 28 degrees of freedom, $t_{0.05} = 2.048$ and $t_{0.005} = 3.047$.

Null hypothesis (H_o:) for paired t test accepted if $t \leq t_{0.05}$; rejected if $t > t_{0.05}$. Sign ignored.

extracts. The analysis in Table 21 indicates that a histidine revertant ratio of 2.0 or more was indicative of a significant increase in the mutagenic activity of crude oil extracts.

The average number of histidine revertants on the strain and solvent control plates were compared by a paired t test to determine if they were significantly different from each other at the 5 per cent probability level. The null hypothesis stated that there was no significant difference between the average number of histidine revertants on strain and solvent control plates for each Salmonella tester strain. The null hypothesis was accepted if the calculated t value was less than or equal to the t value obtained from a standard t table ($t_{0.05}$ = 2.048 with 28 degrees of freedom) and rejected if the calculated t value was greater than that of the t table. The sign of the calculated t value was disregarded.

The results of the paired t test, shown in Table 21, indicate that the number of histidine revertants for Salmonella tester strains TA 100 and TA 98 were significantly lower on solvent control plates than on strain control plates. The R-factor in these strains may have been responsible for the decreased number of spontaneous histidine revertants on solvent control plates containing dimethyl sulfoxide. There was no significant difference in the number of histidine revertants on strain and solvent control plates for the Salmonella tester strains without R-factors. Because dimethyl sulfoxide was added to all mutagenicity test plates in the DMSO extracts of test and control oils, the solvent control was used to determine the histidine revertant ratios of the oil extracts. The solvent controls were used consistently throughout this study, so the differences between strain and solvent controls were not a problem in determining histidine revertant ratios.

DISCUSSION

The results of media evaluation, oil degradation, toxicity studies, mutagenicity testing and data analysis are discussed in this section. The public health implications of this study are also examined.

Media Evaluation

The media evaluation tests provided a basis for selecting appropriate media to optimize the recovery of coliform and heterotrophic bacteria from a variety of aquatic environments. The spread plate technique was used to eliminate the transient warming stress in pour plates (200).

The compositions of the three media tested for recovery of heterotrophic bacteria from river water are compared in Appendix 2. Nutrient agar and plate count agar contain about the same concentration of nutrients, 8.0 and 8.5 g/l of organics, respectively, but the carbon sources are different. Heterotrophic plate count agar contains only 3.5 g/l of organics but has inorganic nutrients that are not present in the other two media.

The results of the media evaluation tests for initial recovery of heterotrophic bacteria from river water agreed with those of other researchers (185, 216, 300). Figures 2 and 3 show that the medium with the lowest nutrient level (HPCA) recovered the greatest number of bacteria. Bacterial recovery was maximized at an incubation temperature of 10 C. Colonial morphology and pigmentation varied with the medium composition. Differences in the recovery of bacteria by the three media were most pronounced when bacteria were initially isolated from river water and were less obvious when they were isolated from oil degradation flasks.

Comparison of five differential media for the recovery of *Escherichia coli* K-12 from high numbers of non-coliform bacteria confirmed that desoxycholate agar and violet red bile agar were superior to eosin-methylene blue agar, tergitol 7-triphenyltetrazolium chloride agar and brilliant green bile agar. The color reaction of the *E. coli* K-12 colonies were exceptionally brilliant on violet red bile agar. This aided in the differentiation of coliform bacteria from heavy background growth and influenced the selection of this medium for the enumeration of *E. coli* K-12 in oil toxicity studies.

Incubation of the violet red bile agar plates at 45 C was an effective means of reducing the number of non-coliform bacteria on the plates and did not reduce the number of *E. coli* K-12 colonies significantly. Although *E. coli* K-12 was not a fecal coliform by definition (6), since it did not produce gas within 24 hours in an elevated temperature test, it was able to grow well at an incubation temperature of 44.5 C which is selective for fecal coliforms. Since coliform recoveries and color reactions were essentially the same at 35 and 45 C, violet red bile agar was well suited for the oil toxicity studies.

Oil Degradation

Oil degradation studies were performed to determine what factors limited the ability of microorganisms to grow on crude oil and to form mutagenic products in the oil. Zobell (383) listed some of the primary limiting factors to microbial growth on oil in water as the absence or low density of oil-degrading microorganisms in the water, unfavorable temperatures, toxic product formation, inhibitory substances, low oxygen tension and insufficient mineral nutrients. The variables tested for limiting effects in this study were incubation time, temperature, nutrient levels and seasonal changes. The relatively low level of non-hydrocarbon nutrients in Chena River water had the greatest effect on microbial growth and mutagen production in Prudhoe Bay crude oil (Figure 4 and Table 15).

Incubation temperatures of 4 and 20 C were chosen for oil degradation and mutagenicity studies, as they were representative of water temperatures in cold and temperate climates, respectively. These temperatures also allowed for the selective growth of psychrophilic and mesophilic microorganisms on the crude oil. The 4 C incubation temperature had a temporary limiting effect of the growth of microorganisms because a longer incubation time was necessary to reach the stationary phase of growth than at 20 C, but the final numbers of heterotrophic bacteria were not substantially reduced by temperature (Figure 4). Figure 5 shows that the weight loss of oil was considerably less at 4 C than at 20 C, however.

Heterotrophic bacteria were chosen to monitor oil degradation processes since the pH of the growth medium was initially adjusted to 7.0, favoring the growth of bacteria over that of yeasts and fungi in the early stages of oil degradation. Bushnell and Haas (56) found that bacteria could grow over a wide pH range on hydrocarbon substrates, but the pH of the aqueous medium has been shown to affect microbial successions on hydrocarbons. The lowering of the pH of the aqueous medium by oil-degrading bacteria growing on oil reported by Reisfeld, et al. (281) is thought to favor the later development of yeasts and fungi (332). Atlas (18) recovered higher numbers of fungi than bacteria from acidic arctic waters that were chronically polluted by oil from natural oil seeps. In general, bacteria are more widely distributed in aquatic environments than yeasts and fungi and presumably have a greater impact on oil in the early stages of degradation (382). Metabolic products formed in the early stages of oil degradation are considered to have the greatest environmental impact, since common metabolites are formed in later stages (225).

Microscopic examination of the contents of oil degradation flasks revealed that bacteria, yeasts and fungi were present, but bacteria were always most numerous. Rod-shaped bacteria were commonly observed at the oil-water interface of emulsified oil droplets. Emulsified oil droplets became heavily colonized by microorganisms after prolonged incubation and were surrounded by a visible layer of growth. These droplets nearly always had a layer of bacteria at the immediate oil-water interface which was covered by a layer of yeasts and fungi, indicating that early colonization of the oil droplet was by bacteria. Bacteria are able to emulsify oil (169, 377, 379) and concentrate at oil-water interfaces (229). Bacteria with these properties may influence the degradation of oil to a greater extent than other microorganisms. The inner portion of the oil droplets was free of microbial growth, indicating that degradation of the droplets occurred on the surface.

The oil degradation studies presented in Figures 4 and 5 established that additional non-hydrocarbon nutrients, especially nitrogen and phosphorus, were needed for optimal microbial degradation of Prudhoe Bay crude oil in Chena River water. Neither the oil or the water supplied sufficient minerals to allow maximum bacterial growth on crude oil. Table 8 shows that the highest total nitrogen and phosphorus levels detected in the Chena River in the first three months of 1976 were 1.03 mg/l and 0.31 mg/l, respectively. An analysis of Prudhoe Bay crude oil by Thompson, et al. (328) showed that it contained 2.3 mg/g of nitrogen and 8.2 mg/g of sulfur. One hundred ml of Chena River water and one gram of Prudhoe Bay crude oil could be expected to supply more than three mg of nitrogen, less than one mg of phosphorus and over eight mg of sulfur. The limiting effect of these levels of non-hydrocarbon nutrients can be seen in Figures 4 and 5. Nitrogen and phosphorus at growth limiting concentrations are rapidly assimilated into microbial biomass and are unavailable for further microbial growth most of the time (369). It is probable that a large portion of the non-hydrocarbon nutrients in crude oil are contained in complex molecules and are not readily available for microbial use, making the available nutrient levels much lower than the total nutrient levels.

Bushnell and Haas (56) reported that 50 mg/l phosphate was required for minimal growth of Pseudomonas on kerosine, but did not find nitrate or sulfate limiting. Mulkins-Phillips and Stewart (255) found that natural degradation of oil in water was limited by low temperature and phosphorus but not by nitrogen. Bunch and Harland (52) showed that 0.5-1.0 g/l of nitrogen and 0.05 g/l of phosphorus was needed for maximum biodegradation of mineral oil in the Beaufort Sea. Atlas and Bartha (23) recovered low levels of total nitrogen and phosphorus from natural seawater. They observed only a three per cent weight loss of crude oil from natural seawater after

18 days at 28 C, but addition of 1.0×10^{-2} M nitrate and 3.5×10^{-4} M phosphate to the seawater increased weight loss to 70 per cent.

The salts medium used in this study supplied 1.25×10^{-2} M nitrate, 1.25×10^{-2} M ammonia and 6.2×10^{-3} M phosphate. Magnesium, sodium, potassium, chloride and sulfate ions were also supplied by the salts medium. Trace elements, iron and other growth factors were supplied by the river water and no attempt was made to optimize these nutrient levels. The increased numbers of heterotrophic bacteria and the greater weight loss of oil in salts-supplemented Chena River water than in raw river water indicate that the salts medium supplied sufficient non-hydrocarbon nutrients to nearly maximize microbial growth on Prudhoe Bay crude oil (Figures 4 and 5).

Most oil degradation studies have used oil concentrations ranging from 0.2-2.0 per cent (376). The concentration routinely tested in this study was one per cent. Table 9 shows that the weight of oil biodegraded was highest at the one per cent level, but the percentage of oil biodegraded was slightly lower than in flasks with less oil. This indicates that the oil degradation flasks were saturated with excess Prudhoe Bay crude oil at the one per cent level. Both the weight and percentage of oil degraded by microorganisms decreased at concentrations above one per cent. Biodegradation of Prudhoe Bay crude oil was maximized at a concentration slightly less than one per cent.

Figure 10 shows that the initial concentration of crude oil added to oil degradation flasks influenced the microbial production of mutagens. Oil concentrations less than one per cent were probably substrate limiting, so that maximum mutagen production was not possible. Oil concentrations above one per cent apparently limited normal microbial metabolism. Edwards (109) demonstrated that high substrate concentrations inhibit biodegradation of substrates. The level of microbiologically-mediated mutagenic activity produced in oil appears to be closely linked to the amount of oil biodegraded, since both oil degradation and mutagen production were highest at an oil concentration of one per cent (Table 9 and Figure 10).

There may be several reasons for the decreased biodegradation of crude oil at high substrate levels. The amount of oil biodegraded within a given time period would be expected to decrease if the substrate concentration exceeded that required to maintain maximum cell density and metabolism. Although the relative percentage of biodegradable substrate in the crude oil is the same, regardless of concentration, the available substrate would be used more rapidly if it was limiting than if it was in excess. High oil concentrations may also be inhibitory to oil-degrading microorganisms because of direct toxicity (72, 151, 164, 330, 352), formation of toxic products (59, 206) and catabolite repression (4, 24, 85). Any or all of these effects may have occurred in the oil degradation flasks at high oil concentrations, as a complete inhibition of oil degradation by microorganisms was observed at a Prudhoe Bay crude oil concentration of five per cent and a severe depression of biodegradation was observed at the two per cent level.

According to Table 9, the weight loss of Prudhoe Bay crude oil from oil degradation flasks due to biodegradation and extraction processes at non-inhibitory concentrations ranged from 49-64 per cent of the initial weight. Up to 29 per cent of this weight loss could be attributed to non-biological processes, such as evaporation and extraction, indicating that 20-35 per cent of the oil was readily available for biodegradation. The remaining residue that was recoverable by Freon^{TM} extraction, consisting of undegraded and partially degraded oil, including mutagenic products, amounted to 36-51 per cent of the oil initially added to the flasks. These results agree closely with those reported by Atlas (18) for microbiological degradation of Prudhoe Bay crude oil. He found a non-biological weight loss of 25 per cent, a microbiological weight loss of 30 per cent and a residue of 45 per cent after incubating one per cent Prudhoe Bay crude oil for 42 days at 20 C with a psychrophilic *Pseudomonas* in seawater supplemented with nitrogen and phosphorus.

Visual differences among replicate flasks were obvious throughout this study. Minor differences in replicate flasks were apparently able to greatly influence the course of oil degradation and the predominant microorganisms in the flasks. It was suspected that the microorganisms that grew fastest in the early logarithmic phase of growth were able to control subsequent microbial successions by competition or by alteration of the environmental conditions in the medium. Since microorganisms are particulate, they may have been unevenly distributed in replicate water samples, allowing different predominant microorganisms to develop in the early stages of oil degradation. Different predominant bacterial isolates were obtained from replicate flasks which were visibly different, indicating that there was a relationship between the kinds of bacteria in the flasks and their appearance. Walker and Colwell (349) noted subtle differences in the types of bacteria isolated from mixed cultures of oil-degrading microorganisms incubated under similar conditions.

Pure cultures of the predominant bacteria isolated from oil degradation flasks grew poorly on fresh crude oil, so mixed cultures were used for oil degradation studies and mutagenicity testing. The predominant isolates tested may have been secondary oil degraders which were incapable of initiating the early steps of hydrocarbon metabolism or they may have lacked essential growth factors required for oil degradation, since the studies were performed in artificial salts medium made with distilled water. There is evidence that natural waters provide microbial growth factors that are not present in artificial media (185). Other studies conducted at the Arctic

Environmental Research Station in College, Alaska showed that pure cultures of bacteria were able to degrade oil much better in natural water samples than in artificial media (D. Badman, personal communication).

The poor growth of pure cultures of bacteria on Prudhoe Bay crude oil was not expected, because predominant isolates from oil degradation flasks were selected for testing. Oil degradation by mixed microbial cultures apparently allows a large number of secondary oil-degraders to develop that are not able to degrade fresh crude oil, but are dependent on degradation products from other microorganisms for growth. It was not possible to test specific isolates for the production of mutagens because of their inability to grow well on the crude oil substrate. A means of separating and identifying primary and secondary oil-degraders may be necessary to determine which group of microorganisms are responsible for the production of mutagens in crude oil.

Several investigators have reported that mixed cultures of microorganisms are capable of degrading more oil than pure cultures (169, 242, 281, 382). Pure cultures of microorganisms lack the diversity of enzymes possessed by mixed cultures and utilize fewer oil components than mixed cultures. Pure cultures form many partial oxidation products, while mixed cultures utilize a larger portion of the oil and degrade partial oxidation products more completely (66, 169).

Pure cultures of microorganisms and pure hydrocarbons have been used in many oil degradation studies to eliminate variables or to study specific biochemical pathways. Such techniques were not employed in this study, since a less accurate picture of actual environmental interactions would be obtained. By using a less defined test system, more variability in the results were expected. The succession of microorganisms growing on crude oil was highly variable, but the factors causing this variability were not defined. It is thought that only a few microorganisms are capable of producing mutagens in crude oil and the expression of mutagenic activity may be dependent on the selective growth of these microorganisms. Seasonal variation in the production of mutagens in crude oil by Chena River water microorganisms supports the hypothesis that specific microorganisms are required for the production of mutagens and that it is not a general phenomenon that can be attributed to all oil-degrading microorganisms.

Walker and Colwell (351) observed that microorganisms obtained from oil-polluted water had a greater oil-degrading potential than microorganisms obtained from unpolluted water. A selective increase in the number of oil-degrading microorganisms in relation to non-oil-degrading microorganisms has been shown to occur in water chronically polluted by oil (30, 167). Varma, et al. (342) used the fluctuation test of Luria and Delbrück (217) to show that wastewater bacteria were acclimated to recalcitrant substrates by mutation selection rather than by enzyme adaptation. The production of toxic or mutagenic components in oil by microorganisms may be controlled in part by selection of mutants capable of forming these products.

The lower ratio of pigmented to non-pigmented bacteria in oil degradation flasks than in raw Chena River water was apparently not due to a decrease in the number of pigmented bacteria, because the pigmented colonies could still be recovered from the flasks on heterotrophic plate count agar at low dilutions. The lower ratio was thought to be caused by the growth of a greater number of non-pigmented than pigmented bacteria on crude oil. The low number of pigmented bacteria may have been due to a selective growth advantage of non-pigmented bacteria on oil or the inability of pigmented bacteria to express pigmentation on hydrocarbon substrates. Evidence for the latter possibility was obtained by observing bacteria plated on heterotrophic plate count agar from oil degradation flasks over a period of time. Upon initial growth at 20 C, few colonies were pigmented, but when the plates were held at room temperature for several days, some of the non-pigmented colonies were able to express pigmentation again. Bacteria isolated from raw Chena River water expressed red, yellow, orange, purple and fluorescent pigments on heterotrophic plate count agar, but pigmented bacteria actively growing on crude oil nearly always expressed yellow pigmentation on heterotrophic plate count agar.

The kinds of microorganisms responsible for oil degradation and mutagen production were not identified in this study. Other studies of arctic and subarctic regions have identified microorganisms common to these areas. The microbial genera isolated from cold regions do not appear to differ much from those in temperate climates. Boyd and Boyd (42, 43) studied the soil and water microorganisms in a subarctic region of Canada and found diverse populations of autotrophic and heterotrophic bacteria, yeasts, fungi, protozoa, etc. They observed that runoff from the soil increased the numbers of bacteria in water considerably, but the numbers of fungi were increased only slightly. The runoff was confined to the top layers of soil because of the permafrost layer underneath.

Fournelle (130) surveyed the types of bacteria in the soil and water of a subarctic region of Alaska and showed that *Achromobacter*, *Alcaligenes*, *Flavobacterium*, *Pseudomonas*, *Bacillus* and *Micrococcus* were common to both the soil and water of the area studied. He found that mesophiles were predominant in subarctic regions and that psychrophiles were in higher numbers than thermophiles. The bacterial genera isolated from an arctic lake by Boyd and Boyd (41) were nearly the same as those isolated from subarctic water by Fournelle (130). *Pseudomonas* spp. were isolated from the lake water most often and Chromobacterium and Streptomyces were also identified. Gaska (135) identified bacterial isolates from the C-100 sampling station on the Chena River where water samples were obtained for this study and found that Pseudomonas, Acinetobacter, Flavobacterium, Arthrobacter and several members of the families Pseudomonadaceae and Vibrionaceae were present at this site at the time of sampling.

Yeasts may also have an important role in the microbial degradation of oil in subarctic waters. Sinclair and Stokes (304) isolated obligately psychrophilic yeasts of the genera Candida and Torulopsis from polar regions. Meyers and Ahearn (242) tested over 700 yeast isolates from fresh and salt water and soil for their ability to degrade hydrocarbons. They found that the predominant yeasts capable of degrading petroleum hydrocarbons included members of the genera Pichia, Debaromyces, Candida, Torulopsis, Rhodotorula and Trichosporon.

Ivarson (181) identified 11 genera of fungi in permafrost soils in Canada; Penicillium, Mucor, Pseudogymnosascus Oidiodendron, Philalophora, Mortierella, Pestalotia, Trichoderma, Humicola, Crysosporium and Cladosporium. Table 2 shows that several of these genera are capable of oxidizing hydrocarbons.

Chocolate mousse (a water-in-oil emulsion) formation occurred in a few oil degradation flasks. These flasks were not used for oil degradation studies or mutagenicity testing because microbial degradation and emulsification of the crude oil was noticeably decreased. Chocolate mousse formation was minimized by thoroughly washing and rinsing the glassware by hand. A higher incidence of chocolate mousse formation was seen in machine-washed glassware, possible due to incomplete rinsing of detergent residues from the flasks.

Toxicity Studies

The observation of primary importance in oil toxicity studies was that microbial degradation of Prudhoe Bay crude oil was necessary to increase its toxicity to *Escherichia coli* K-12. The bacteriocidal effect of the degraded oil on *E. coli* K-12 (Figure 7) is of interest, since most of the toxic effects of oil on bacteria have been reported to be bacteriostatic in nature, except for high concentrations of volatile hydrocarbons (330).

Figure 7 shows that the die-off of *E. coli* K-12 was greatest during the logarithmic phase of growth of heterotrophic oil-degrading bacteria. It was suspected that the appearance of toxic effects in the oil was paralleled by an increase in its mutagenic activity. Many mutagenic agents are acutely toxic to bacteria and a certain percentage of kill is necessary to mutate the maximum number of bacteria with such chemicals (158). Mutagenicity tests were not performed on oil extracts from toxicity study flasks, but it was possible to detect acute toxicity of Prudhoe Bay crude oil to E. coli K-12 within the same incubation time that mutagenic activity could be detected in oil extracts for mutagenicity testing. Both effects were detected within one week at 20 C in salts-supplemented Chena River water (Figures 7 and 9).

The decrease in the number of E. coli K-12 in the presence of Prudhoe Bay crude oil biodegradation intermediates was not as large as expected. The toxicity of the oil was transitory, apparently occurring only when certain microorganisms and/or metabolic products were concentrated enough to exert lethal effects on E. coli K-12. A similar study performed with Black Hollow, Colorado crude oil also showed that microbial degradation was necessary before the oil became lethal to E. coli K-12, but 2.5 weeks of incubation with a crude oil adapted culture at 25 C was required before a significant die-off of E. coli K-12 was observed. Mutagenic activity was not detected in Black Hollow crude oil incubated in a similar set of oil degradation flasks under the same conditions until the third week, indicating that there may be a close relationship between the acute toxicity and mutagenic activity of biodegraded crude oil.

A non-oil-degrading bacterium was chosen for the oil toxicity studies, because oil-degrading bacteria may be more tolerant to the toxic effects of their own metabolic products. The acute toxicity of the crude oils to bacterial genera in the family Enterobacteriaceae, such as Escherichia and Salmonella was also being tested. These studies indicated that Black Hollow crude oil and Prudhoe Bay crude oil were probably not toxic enough to the Salmonella tester strains to affect the results of the Ames test. Non-oil-degrading bacteria have been used in other studies to monitor the toxic effects of oil (59, 151). The toxicity of oil biodegradation products to bacteria in natural waters are apparently negligible due to dilution effects. Bartha and Atlas (30) stated that large amounts of water and mixed microbial populations prevent the buildup of high concentrations of toxic products in natural waters in most cases. The toxic effects of oil pollutants on aquatic organisms may be more prevalent in waters with slow turnover rates.

The presence of a protozoan predator in the Chena River complicated the oil toxicity studies, because the toxic effects of Prudhoe Bay crude oil on E. coli K-12 were masked by the predatory activity in the water which caused a greater decrease in the E. coli K-12 population than the oil. The agent responsible for the rapid decline in E. coli K-12 numbers in raw Chena River water was not identified for some time, but the observation that it could be eliminated by autoclaving or filter-sterilizing the river water suggested that the lethal factor was a heat-labile, biological factor greater than 0.22 micron. Undegraded crude oil and sterile river water had no adverse effects on the survival of E. coli K-12, showing that chemicals present in river water or fresh crude oil were not responsible for the decline in the number of *E. coli* K-12 in the test flasks. Since the *E. coli* K-12 declined faster in flasks without oil than in flasks with oil, the oil appeared to have an inhibitory effect on the lethal factor, further indicating its biological nature. The selection for and observation of a protozoan actively ingesting *E. coli* K-12 confirmed that a biological factor larger than 0.22 micron was responsible for the rapid decline of *E. coli* K-12 at high population densities.

A comparison of Figures 6 and 7 shows that the protozoan predator was eliminated from crude oil adapted cultures of river water microorganisms. The reason for the disappearance of the predator from adapted cultures is not known, but may have been caused by dilution upon transfer of the culture to fresh medium or toxic products. By using adapted cultures, the effects of crude oil on the *E. coli* K-12 population could be observed during microbial degradation, free from interfering influences.

Mitchell (244) and Hendricks and Morrison (161) suggested that protozoans were a possible cause of the decline of coliform bacteria in natural waters. Danso and Alexander (88) studied the predation of *Rhizobium* cultures by protozoans in soil and water. They found that high prey densities of 10^8-10^9 cells/ml increased the numbers of protozoan predators substantially, but that lower prey densities of

10^6-10^7 cells/ml did not increase the protozoan populations significantly.

A predator-prey relationship similar to that described by Danso and Alexander (88) can be seen in Figure 6. The initially high number of E. coli K-12 added to the test flasks allowed rapid development of the protozoan predator before the heterotrophic bacteria in the river water reached the stationary phase of growth. In the absence of oil, the heterotrophic bacteria declined along with the E. coli K-12 in test flasks of Chena River water, indicating that the predation was not specific for E. coli K-12. As the bacterial numbers declined in the flask, the rate at which they were killed also declined, suggesting that a high population density of the prey is necessary to maintain the predator at a high cell density. Predation of the oil-degrading bacteria probably occurred in the test flasks containing oil, but they did not decrease in number because they were able to grow faster on the oil than they were eliminated by the protozoan. The E. coli K-12 population did decline in the flask containing oil, since it was unable to grow on the oil and replace the numbers lost. The fluctuation of oil-degrading bacterial numbers in the stationary phase of growth on oil in natural water samples observed in other oil degradation studies may be caused by similar predator-prey relationships (168, 349).

The slower decline of E. coli K-12 in flasks with oil than in flasks without oil appeared to be caused by inhibitory effects of the oil on the protozoan predator, but interference by the large number of oil-degrading bacteria can not be ruled out. The inhibitory effect of the oil decreased with time and may have been caused by low-boiling, volatile components in the oil which delayed the development of the predator until they evaporated. Jacobs (182) observed an increase in bacterial numbers and a decrease in protozoan numbers in greenhouse soils treated with naphthalene. After most of the naphthalene had evaporated from the soil, the protozoan numbers increased rapidly. The naphthalene derivatives of crude oil may have exerted a similar influence on the protozoans in the toxicity test flasks containing oil. Since one protozoan type appeared to predominate over others, it may have been more resistant to the toxic effects of the oil.

Mutagenicity Testing

The mutagenicity testing procedure employed in this study was patterned after the Ames test variation employed by Durston and Ames (107) which used live mice to enzyme activate promutagens to mutagens. Enzymes produced by microorganisms during the microbial degradation of crude oil constituted the enzyme activation system for this study. One of the main objectives of this study was to determine if microbial enzymes could activate chemicals to mutagens in a manner analogous to that of mammalian enzymes.

The greatest problem encountered in mutagenicity testing of extracts from oil degradation flasks was the variability in mutagenic activity of extracts from replicate flasks (Table 16). In an attempt to minimize this variability, triplicate flasks were incubated under the same conditions and their extracts were combined for mutagenicity testing. By combining the extracts, a more discriminatory test of the potential of river water microorganisms to produce mutagens in crude oil was possible, because biases due to single flask variations were eliminated. A positive test for mutagenic activity in combined extracts indicated that a majority of the extracts contained some mutagenic activity, since a majority of extracts with no mutagenic activity would presumably dilute the mutagenic activity of a single extract to an undetectable level.

Table 16 shows that the mutagenic activity of uncombined replicate oil extracts incubated under the same conditions were so inconsistent that a positive or a negative test for mutagenic activity could be obtained, depending on which extract was chosen for mutagenicity testing. A false impression of the potential of river water microorganisms to produce mutagens in crude oil could easily be obtained by testing single extracts. It is possible that a false test could be obtained by testing combined extracts, but the probability of this occurring should be less frequent, as a greater number of variables are tested.

An attempt was made to stabilize the microbial populations in oil degradation flasks by adapting them to growth on crude oil. It was hoped that a stable culture of oil-degrading microorganisms would produce consistent mutagenic activity in crude oil under fixed conditions so that the effect of variables on the production of mutagens in oil could be studied semi-quantitatively. The adapted culture studies were only partially successful, as the variability among replicate flasks was reduced, but not eliminated, and the mutagenic activity present in the flasks was not consistent. Semi-quantitative testing of the mutagenic activity of oil extracts under controlled variable conditions was complicated by seasonal variations in the river water and was successful only when the microbiologically-mediated mutagenic activity of the extracts was at a high level in the spring and fall. Figures 9, 10, 11 and 12 show the results of semi-quantitative testing of oil extracts during times of peak mutagenic activity.

Seasonal effects on the ability of river water microorganisms to produce mutagens in crude oil were linked to surface water runoff into the Chena River. The most convincing evidence of this was the dramatic increase in microbiologically-mediated mutagenic activity detected in Prudhoe Bay crude oil incubated with Chena River water at the time of ice breakup in May (Figures 13-17). The greater ability of river water microorganisms to produce mutagens from crude oil during periods of increased surface water runoff into the river may be due to higher numbers of soil microorganisms in the water capable of producing mutagens from oil and/or an influx of nutrients from the soil required for the microbial production of mutagens. The total number of heterotrophic bacteria recovered from the river water did not appear to correlate with the increases in microbiologically-mediated mutagenic activity in crude oil observed at various times of the year, possibly because of greater dilution of the microorganisms during periods of increased surface water runoff.

Seasonal variations in bacterial populations have been observed in other studies of natural waters (115, 260, 302). Ezura, et al. (115) reported that surface water runoff was probably responsible for increased numbers of terrestrial microorganisms in seawater at certain times of the year. Morrison and Fair (249) found that the greatest increase in total bacterial numbers in a mountain stream occurred after a rain. Walker and Colwell (349) observed an increase in the oil degrading potential of water samples taken from the Chesapeake Bay after a heavy rainstorm. They also noted that the water temperature had a selective effect on the kinds of microorganisms present in the water at various times of the year and concluded that utilization of petroleum hydrocarbons by microorganisms at low temperatures was a function of the types and numbers of microorganisms in the natural environment and seasonal fluctuations in the microbial populations. Floodgate (126) reported similar effects on oil degradation in natural waters and proposed that the rate of microbial degradation of oil in water varies with the chemical complexity of the oil, the type and number of microorganisms present and the environmental conditions. There is evidence that mesophilic temperatures are highly selective for oil-degrading microorganisms (83, 302, 359).

Table 15 shows that the amount of nutrients available to microorganisms during oil degradation not only affects the amount of oil degraded, but the products formed, as well. Addition of non-hydrocarbon nutrients to the water samples was necessary for the production of microbiologically-mediated mutagenic activity in the crude oils tested in this study. Although nitrogen and phosphorus appear to have the greatest effect on oil degradation (25), other nutrients may also influence the production of mutagens in oil. Biodegradable analogs of recalcitrant molecules have been used to enhance their co-metabolism in the presence of pure and mixed cultures of microorganisms. The biodegradable analogs stimulate the production of enzymes required for co-metabolism in microorganisms much better than chemically unrelated substrates such as glucose (32, 172, 278).

As long as the essential non-hydrocarbon nutrients are in excess, crude oils contain the carbon and energy sources necessary for the growth of oil-degrading microorganisms. Easily degraded alkanes are present to support rapid cell growth. Enzyme inducers and biodegradable analogs of recalcitrant molecules are present to regulate the synthesis of enzymes required for the biodegradation of certain components of the oil. Table 5 shows that microorganisms are able to biosynthesize most of the necessary structural components, amino acids and enzymes for cellular growth from alkanes and aromatics in petroleum, allowing them to degrade the oil components that are more resistant to biodegradation. Partial oxidation of recalcitrant molecules, such as polynuclear aromatic hydrocarbons, may result in the formation of toxic or mutagenic products.

Figure 5 and Table 15 show that the percentage of Freon^{TM}-extractable crude oil was lower in test than in control flasks at both 4 and 20 C before microbiologically-mediated mutagenic activity was detected in the oil. These results indicate that microbial degradation of the oil was necessary for the production of microbiologically-mediated mutagenic activity. Although there seems to be a connection between microbial degradation of oil and mutagenic activity, weight loss was not as important for the production of non-biologically-mediated mutagenic activity. Weight loss of oil is probably the least reliable indicator of non-biological or microbiological production of mutagens, as Table 3 shows that the weight of asphaltene and NSO fractions of Prudhoe Bay crude oil are increased after microbial degradation. The incorporation of nitrogen, sulfur or oxygen atoms into hydrocarbons actually increases the weight of partially oxidized compounds.

It was possible to distinguish between the non-biologically-mediated and microbiologically-mediated mutagenic activity of crude oil extracts by means other than weight gain or loss. In the majority of cases, mutagenic activity was higher in microbiologically-degraded oil than in control oil. In addition, both frameshift and base-pair substitution mutagens were detected by the Salmonella tester strains in oil after microbial degradation, but only frameshift mutagens were detected in control oil extracts. The production of base-pair substitution mutagens in Prudhoe Bay crude oil appeared to be dependent on microbial degradation processes.

Salmonella tester strains TA 1537, TA 1538 and TA 98, which are sensitive to frameshift mutagens, responded positively to non-biologically-mediated and microbiologically-mediated mutagenic activity in control and test oil extracts, respectively (Tables 12 and 13). Salmonella tester strains TA 100 and TA 1535 are both sensitive to base-pair substitution mutagens, but TA 100 was the only strain that reacted positively to the microbiologically-mediated mutagenic activity in test oil extracts. Since both of these tester strains contain the same histidine mutation, the R-factor in TA 100 was apparently responsible for the increased sensitivity of this strain to the mutagens in crude oil. Figures 11 and 12 show that TA 1535 did not give a consistent dose response over a wide range of oil concentrations and was not able to detect mutagens within the concentration range tested. Salmonella tester strain TA 100 appears to be able to detect mutagens produced by microbial enzyme activation, exclusively, and may be the most useful tester strain for studying microbiological interactions with chemicals in the environment.

The Salmonella tester strains are generally quite specific for certain types of mutagens (Appendices 4 and 6), but some mutagens are capable of inducing both frameshift and base-pair substitution mutations in the strains (46). The Salmonella tester strains were not necessarily detecting different mutagens in microbiologically-activated crude oil, but a clear distinction could be made between non-biologically-mediated and microbiologically-mediated mutagenic activity on the basis of the type of mutagens detected.

The mechanism by which mutagens are produced by microorganisms may be direct or indirect. Direct mutagen production by microorganisms may be accomplished by formation of mutagenic metabolic intermediates during biodegradation of oil. Indirect mutagen production by microorganisms may be accomplished by microbial degradation of masking components in the oil, making the remaining oil more susceptible to non-biological activation. The mechanism by which frameshift mutagens are formed in oil may be quite similar for non-biological and microbiological activation processes. Although it is not certain which mechanism is used by microorganisms to produce frameshift mutagens in crude oil, microbial mediation is required in both cases. Base-pair substitution mutagens were formed in crude oil by direct mutagen production, entirely.

Tables 19 and 20 show that the mixed microbial populations in Chena River water noticeably decreased the non-biologically-mediated mutagenic activity in the aromatic fraction of Prudhoe Bay crude oil within one week. Figure 9 indicates that microbiologically-mediated mutagenic activity was more resistant to microbial degradation, since both frameshift and base-pair substitution mutagens remained at high levels of activity for two weeks or more. These results suggest that the frameshift mutagens formed in crude oil by non-biological activation were different from those formed by microbiological activation.

Figure 9 shows that both activation and inactivation of mutagens occurred in Prudhoe Bay crude oil during its biodegradation by microorganisms. These two processes may have occurred at different times during incubation or simultaneously, but at different rates. Table 14 shows that microbiologically-mediated mutagenic activity did not always appear in the oil at the same time, but that it could remain at detectable levels for three to four weeks. It is possible that the same microorganisms are responsible for activation and inactivation of mutagens. Ciegler, et al. (74) found that Aspergillus flavus could both synthesize and degrade the mutagenic carcinogen, aflatoxin B_1. The ability of microorganisms to activate and inactivate mutagens is analogous to the ability of mammalian enzyme systems to form carcinogens and then detoxify them (121, 263). Most biological systems have some means of detoxifying harmful chemicals.

Only frameshift mutagens were detected in the aromatic fraction of Prudhoe Bay crude oil after incubation of the various fractions with raw and sterile river water (Tables 19 and 20). The absence of base-pair substitution mutagens in the fractions and the rapid destruction of frameshift mutagens in the aromatic fraction in the presence of river water microorganisms indicate that these mutagens were not microbiologically-mediated. The mutagens may have been present in the oil prior to fractionation and were expressed only after masking fractions of the oil were removed. Back-addition of fractionated oil components to the aromatic fraction partially or completely masked expression of mutagenic activity (Table 20).

An alternative explanation for the appearance of frameshift mutagens in the aromatic fraction is that they were formed by non-biological oxidation during fractionation or incubation. The necessity of incubating fresh Prudhoe Bay crude oil in control flasks for the production of non-biologically-mediated mutagenic activity supports this hypothesis. The mutagen concentrations in fresh Prudhoe Bay and Black Hollow crude oils were too low to be detected by the Ames test and non-biological alteration of the oil components was necessary to produce a detectable level of mutagenic activity in the aromatic fraction of the oil. Crude oil apparently contains a higher concentration of promutagens requiring activation to mutagens than fully active mutagens. Fractionation of the oil apparently rendered the aromatic fraction more susceptible to non-biological oxidation, as mutagens were produced more consistently in the aromatic fraction than in whole crude oil in control flasks.

Table 20 shows that microbiologically-mediated mutagenic activity was detected in whole crude oil by Salmonella tester strain TA 100, but that no microbiologically-mediated mutagenic activity was present in fractionated crude oil. These results suggest that whole crude oil was necessary for the microbial production of mutagens. The fractionated crude oil may have lacked components required for mutagen production or the microbial populations may have been less stable on substrates other than whole crude oil.

It is not known if crude oil fractions other than the aromatics contained microbiologically-mediated mutagenic activity, since test oil extracts of crude oil with known mutagenic activity were not fractionated for mutagenicity testing. A means of preventing non-biological oxidation of the aromatic fraction during fractionation may be necessary to distinguish between biological and non-biological activation of aromatics to mutagens.

The ability to detect non-biologically-mediated mutagenic activity in Prudhoe Bay crude oil, but not in Black Hollow crude oil, suggested that there were major differences in the compositions of the two oils. A comparison of fractional distillation analyses of these oils revealed that the main difference in their composition was that Prudhoe Bay crude oil had a much higher percentage of asphaltic residuum than Black Hollow crude oil (328, 358). Prudhoe Bay crude oil may contain more promutagens in the residuum than Black Hollow crude oil. Table 6 shows that the carcinogenic activity of crude oil is located in the high-boiling fractions of petroleum. Most carcinogenic polynuclear aromatic hydrocarbons are frameshift mutagens in the Ames test (11).

The mutagens produced by non-biological and microbiological activation have not been chemically characterized, but some idea of their general character may be derived from their chemical properties. It is not certain how the mutagens were formed in control oil flasks by non-biological processes. Chemical oxidation or autooxidation are the most likely means by which this could be accomplished. The delayed appearance of frameshift mutagens in control oil is most consistent with a free-radical mechanism of activation followed by autooxidation with atmospheric oxygen. Some of the products formed in oil by autooxidation, such as peroxides and lactones (206), are potentially carcinogenic to mammals (340). Autooxidation of the hydrocarbons in crude oil may have been catalyzed by organometallic compounds in the oil or by light (132), since the flasks were incubated under incandescent lighting. Lacaze and deNaide (205) showed that the toxicity of crude oil could be increased by natural or artificial light. Inhibitors of free-radical chain reactions in the oil, such as phenols, organic amines, or sulfoxides (132), may have been removed by dissolution into the aqueous phase due to their polar nature. These compounds may have been responsible for masking effects of certain fractions on the production of mutagens in other fractions. Table 20 shows that the non-hydrocarbon asphaltene and NSO fractions had the greatest masking effects on the expression of mutagenic activity in the aromatic fraction of Prudhoe Bay crude oil.

The appearance of non-biologically-mediated mutagenic activity only in the aromatic fraction of crude oil may be explained by the fact that hydrogen atoms on benzylic carbons are most easily removed by catalysts to form free-radicals, favoring the formation of stable, potentially mutagenic aromatic hydroperoxides (132, 163, 250). Updegraff and Bovey (334) showed that microorganisms were capable of degrading organic hydroperoxide intermediates formed by free-radical mechanisms. This may explain why non-biologically-mediated mutagens disappeared from oil degradation flasks faster than microbiologically-mediated mutagens in the presence of microorganisms.

It was suspected that polar non-hydrocarbons formed during microbial degradation of petroleum hydrocarbons would be more mutagenic than the parent hydrocarbons, so the solvents used for extraction were chosen to favor the recovery of relatively polar compounds. A non-selective, general solvent was used to recover as much of the oil from water as possible. The solvent chosen for this purpose, 1,1,2-trichloro-1,2,2-trifluoroethane, is a non-specific solvent capable of extracting hydrocarbons, non-hydrocarbons and detergents from water, according to Standard Methods (6). An undissolved "rag" of oil has been observed after extraction of oil from water with this solvent. It may not extract as much asphaltic material from the oil as solvents like benzene (227).

The second stage of extraction with dimethyl sulfoxide was much more selective for specific components in the oil extracts.

Only a small amount of the oil extracts dissolved in dimethyl sulfoxide, turning the colorless solvent light yellow. Extracts of microbially degraded oil were darker yellow than control oil extracts. The dimethyl sulfoxide extract of the saturate fraction of Prudhoe Bay crude oil was colorless, but extracts of the aromatic and NSO fractions turned the solvent yellow.

Dimethyl sulfoxide is classified as an aprotic solvent which is able to solvate both organic and inorganic chemicals and can be dissolved in both aqueous and organic solvents (250). Cations are solvated more strongly than anions in aprotic solvents, which is the opposite of protic solvents like alcohols and water. Bases become more basic and nucleophiles become more nucleophilic in dimethyl sulfoxide. Because dimethyl sulfoxide contains two highly electronegative atoms, sulfur and oxygen, it was expected to be somewhat selective for polar compounds in oil.

Some mutagens may have been lost by the choice of solvents, particularly in the asphaltene and NSO fractions which contained a higher percentage of solvent insoluble compounds. The solvents used did allow detection of mutagens in a relatively small portion of the crude oil, since very little of the Freon^{TM}-extractable oil dissolved in the dimethyl sulfoxide and only one-twentieth of the dimethyl sulfoxide extract was added to each mutagenicity test plate. The dimethyl sulfoxide extract allowed better dissolution of oil components in the aqueous top agar of the mutagenicity test plates. Since the dimethyl sulfoxide dissolved in the aqueous medium, it was able to select components from the oil extracts that were most compatible with an aqueous system. Most of the oil components in the extracts formed a cloudy emulsion in the top agar, but some of the components formed a slick on top of the agar.

The mutagens in oil extracts were stable for several weeks in oil degradation flasks and were not destroyed by the 70 C temperature they were subjected to in the Freon^{TM}-extraction procedure. The requirement for additional nitrogen and phosphorus in oil degradation flasks for the detection of microbiologically-mediated mutagenic activity in crude oil strongly implicates the non-hydrocarbon compounds as mutagens in the oil extracts. Nitrogen heterocycles, nitrosamines and other nitrogen containing compounds are mutagenic in the Ames test (13). Oxygen containing compounds, such as epoxides, quinolines and aldehydes are also mutagenic to the Salmonella tester strains. The nitrogen and oxygen containing compounds may be formed during microbial metabolism of the oil.

Different types of mutagens and/or carcinogens are apparently produced in oil by microbial and mammalian enzymes. Carcinogenicity tests on animals have shown that the polynuclear aromatic hydrocarbons are primarily responsible for the carcinogenic activity of oil (35). Many carcinogenic polynuclear aromatics are degraded by microorganisms in aquatic environments, so they may constitute only a minor part of the microbiologically-mediated mutagens in oil (93, 305, 384). Liver microsomal enzymes may be necessary for the activation of these components (Figure 1).

Salmonella tester strain TA 1537, which is most sensitive to carcinogenic polynuclear aromatic hydrocarbons (Appendix 4), gave the weakest positive response to the mutagens in microbiologically degraded crude oil. Salmonella tester strains TA 98 and TA 1538, which are most sensitive to aromatic nitroso compounds, gave relatively strong responses to the microbiologically-mediated mutagens in crude oil. This was expected, since nitrogen and phosphorus salts were required for the production of mutagens in oil by microorganisms. Salmonella tester strain TA 100, which is sensitive to alkylating agents that cause base-pair substitution mutations gave relative strong responses to the mutagens in oil. Frameshift mutagens have been implicated as potential carcinogens more often than base-pair substitution mutagens, however (8), so the base-pair substitution mutagens may have little carcinogenic activity.

Whole crude oil was initially added to oil degradation flasks by weight so that the impact of the volatile components of the crude oil would be present at the beginning of the incubation period. Accurately weighing the oil presented a problem because weight was lost from the oil during the weighing procedure. It was determined that approximately ten milligrams of the oil evaporated within the first minute after addition to the aqueous medium in the oil degradation flasks. Weighings were completed within one minute, so ten milligrams (one per cent) of the total weight added to each flask represents a maximum weight loss during weighing.

The biochemical pathways proposed for the microbial metabolism of hydrocarbons do not indicate that toxic or mutagenic products are formed by these processes. For this reason, it is suspected that mutagen production by microorganisms does not proceed by normal biodegradation pathways. Co-metabolism, transformation and nonenzymatic conversion of crude oil components are possible means of forming stable, mutagenic intermediates. Many of the compounds formed by these processes are dead-end products that are resistant to further microbial degradation but are still able to interact with biological systems (1, 193).

Liver microsome activation of promutagens to mutagens was not used in this study, since the purpose of the study was to investigate the possibility that microbial enzymes could activate promutagens in a manner similar to mammalian enzymes. The results show that microorganisms are capable of activating chemicals in crude oil to mutagens. Since the Ames test is known to detect a higher percentage of potentially carcinogenic mutagens if liver microsome activation is employed in the test system (46), higher levels of mutagenic activity may have been demonstrated in this study if a combined microbial-mammalian microsome activation system had been employed in mutagenicity testing.

Since microbiologically-mediated mutagenic activity was detected in two different crude oils in two geographically distinct areas, the results appear to be applicable to other waters, as well. Caution must be exercised in extrapolating the results from one area to another, however. Although mutagens were formed during microbial degradation of crude oil from Colorado and Alaska, base-pair substitution mutagens were detected in Prudhoe Bay crude oil, but not in Black Hollow crude oil. This may have been due to differences in the oils, the microorganisms or other factors at the two locations. Markow (228) reported that the water sampling site in Colorado was affected by surface water runoff, so the sampling sites in both Colorado and Alaska were subjected to introduction of microorganisms and/or nutrients from soil that may have influenced the production of mutagens in the crude oils by microorganisms. Voll, et al. (345) conducted a study similar to this one in estuarine waters, but they were unable to demonstrate, conclusively, that mutagens were produced in oil by the microorganisms present in the water.

Natural waters are subject to many more variables than were examined in this study and crude oil would not be exposed to the density of microorganisms in oil degradation flasks under natural conditions, but the laboratory data may have predictive value for determining the ability of a given aquatic environment to produce mutagens from crude oil. The results of this study indicate that the conditions favoring oil degradation also favor the production of mutagens in oil by microorganisms. Both oil degradation and mutagen production were greatest in oil degradation flasks incubated at moderate temperatures with an excess of non-hydrocarbon nutrients and were subject to seasonal variations in the river. These results suggest that microbiological activation of oil to a mutagenic state is most likely to occur in natural waters when water temperatures are moderate, nutrient levels are high, surface water runoff is significant and chronic oil pollution is present. Waters with slow turnover rates, such as holding ponds, lakes, etc., may be more conducive to the production of mutagens in oil by microorganisms than rivers, since higher concentrations of microorganisms and nutrients may be available for a longer period of time.

Data Analysis

Oil extracts yielding histidine revertant ratios of 2.0 or more with any *Salmonella* tester strain expect TA 1535 were considered to contain significant mutagenic activity (Table 21). Oil extracts yielding histidine revertant ratios less than 2.0 with all five *Salmonella* tester strains contained essentially no mutagenic activity, since a ratio of 2.0 indicated only a marginal increase of histidine revertants. Histidine revertant ratios much less than 1.0 may indicate that the test oil extract was too toxic to the Salmonella tester strains at the concentration tested.

The method used to determine the mutagenic activity of oil extracts was also designed to determine the means by which the mutagens were produced in the oil. The expression for total mutagenic activity was based on the assumption that the microbiologically-mediated mutagenic activity of a test oil extract would always be greater than the non-biologically-mediated mutagenic activity of the corresponding control oil extract. The formulae worked quite well for crude oil extracts, but broke down when crude oil fractions were tested, since extracts of aromatic controls always contained more mutagenic activity than those of aromatic tests. This was apparently caused by the ability of microorganisms to inactivate non-biologically-mediated mutagenic activity in the aromatic fraction. In cases where non-biologically-mediated mutagenic activity was greater than microbiologically-mediated mutagenic activity, the expressions for total and microbiologically-mediated mutagenic activity became meaningless, because the microbiologically-mediated mutagenic activity could not be distinguished from the non-biologically-mediated mutagenic activity and the total mutagenic activity was equal to the non-biologically-mediated mutagenic activity.

Public Health Implications

Bartha and Atlas (30) suggested that microbiological degradation of oil spills in the ocean could be stimulated by adding oleophilic nitrogen and phosphorus fertilizers to the oil slicks. They stated that such practices were a feasible means of removing oil from water, but proposed that several safety criteria, similar to those used to evaluate microbial insecticides, be used to evaluate these practices before adopting them for general use. The proposed safety criteria were as follows:

1. Are hydrocarbon degrading microorganisms free of pathogenicity to man, animals and plants?
2. Are any intermediates of oil biodegradation unacceptably toxic?
3. Will oxygen depletion caused by stimulated oil metabolism injure or kill aquatic life?
4. Does stimulated oil biodegradation increase hydrocarbon contamination in the food chain?
5. Are any of the fertilizers toxic or will they cause eutrophication problems?

This study was designed to test the second safety criterion. Stimulation of natural biodegradation processes should normally pose no threat to the environment, but if such practices increase the burden of toxic products in water to dangerous levels, this may not be advisable.

The Ames test was chosen to assay for mutagenic activity in oil extracts on the basis of its sensitivity and selectivity for mutagens in low concentrations; not its predictive value for the detection of potential carcinogens. In order to make any judgments on the carcinogenicity of oil extracts containing microbiologically-mediated mutagenic activity, it would be necessary to run animal carcinogenicity tests in conjunction with the Ames test. Because not all mutagens are carcinogenic (131) and carcinogenicity tests were not performed in this study, no conclusions can be made about the public health hazard of microbiologically-mediated mutagens in oil.

It is possible that microbial degradation products of oil in water may present a health hazard to aquatic or terrestrial organisms that ingest them, since both the acute and chronic toxicity of crude oil to bacteria was increased after exposure to oil-degrading microorganisms. The microbial degradation products may not be carcinogenic in themselves, but may be activated to carcinogens by other organisms. Large amounts of oil and fertilizers enter the rivers and oceans of the world each year, thus supplying the elements necessary for microbial production of toxic metabolites from oil in natural waters. Factors such as low temperatures and dilution may minimize the production of toxic chemicals by microorganisms. The results of this study indicate that microbiologically-mediated mutagens were produced in higher concentration than non-biologically-mediated mutagens. This effect may be reversed in waters with low temperatures and nutrient levels. Suess (320) found that degradation of polynuclear aromatic hydrocarbons in the ocean occurred primarily in the upper layers of the water by photooxidation.

Several studies have shown that a potential health hazard exists in water exposed to chronic pollution by oil or other chemicals. Water samples from the lower Mississippi River obtained near chemical plants and oil refineries were shown to increase the number of frameshift mutations in Salmonella tester strains when assayed by the Ames test (271). Page, et al. (267) discovered increased cancer mortality rates in Louisiana parishes that obtained their drinking water from the lower Mississippi River rather than from wells. Simmon and Tardiff (303) found that drinking water extracts concentrated by reverse osmosis were mutagenic in the Ames test. The U. S. Environmental Protection Agency (338) has identified a number of suspected carcinogens, including polynuclear aromatic hydrocarbons, in drinking water concentrates. Mutagens were detected in used crankcase oils activated with trout liver microsomes by Payne, et al. (270).

In many instances, the health hazards of chemicals have been exaggerated because the detoxification mechanisms of man and animals have not been taken into account. Cornfield (81) suggested that a risk assessment of suspected carcinogens should be performed before completely banning them from use. This approach may be more reasonable, as it advocates the establishment of threshold limits for chemicals that have substantial benefits and relatively low carcinogenic potentials. The National Academy of Sciences (259) is presently evaluating the health hazard of the weak carcinogen, saccharin, on a risk-benefit basis.

SUMMARY AND CONCLUSIONS

In summary, the significant observations of this study are as follows:

1. The mutagenic activity of crude oil was increased by both non-biological and microbiological processes. Activation of mutagens in crude oil by non-biological processes produced frameshift mutagens only, while microbiological processes produced both frameshift and base-pair substitution mutagens.
2. Microbiological activation of crude oil to a mutagenic state was demonstrated at 4, 20 and 25 C. A longer incubation time was required to detect mutagenic activity at 4 C than at 20 or 25 C.
3. Addition of nitrogen and/or phosphorus supplements to the growth medium was necessary to detect microbiologically-mediated mutagenic activity in crude oil. Oil degradation, heterotrophic bacterial numbers and mutagenic activity were greatest when excess nitrogen and phosphorus were present in the growth medium during microbial growth on crude oil.
4. Non-biologically-mediated mutagenic activity in crude oil was detected by Salmonella tester strains TA 1537,

TA 1538 and TA 98, which are all sensitive to frameshift mutagens. Microbiologically-mediated mutagenic activity in crude oil was detected by Salmonella tester strains TA 100, TA 1537, TA 1538 and TA 98. Tester strain TA 100 is sensitive to base-pair substitution mutagens and detected mutagens in crude oil only after microbial degradation of the oil had occurred.

5. The mutagenic activity of whole crude oil was increased by microorganisms, but the mutagenic activity of crude oil fractions was decreased by microorganisms, showing that microorganisms from river water were capable of increasing or decreasing the mutagenic activity of oil.
6. Crude oil concentrations of one per cent (w/v) in aqueous media yielded the highest levels of microbiologically-mediated mutagenic activity.
7. The aromatic fraction contained all of the non-biologically-mediated mutagenic activity detected in fractionated crude oil.
8. Weight loss of solvent extractable oil was observed in conjunction with the detection of microbiologically-mediated mutagenic activity. A similar, but much smaller weight loss preceded the appearance of non-biologically-mediated mutagenic activity.

9. Increased surface water runoff into the Chena River appeared to increase the ability of river water microorganisms to produce mutagens in crude oil.
10. The ability of freshwater microorganisms to increase the mutagenic activity of crude oil was demonstrated in water samples from Colorado and Alaska.
11. Crude oil was toxic to a non-oil-degrading Escherichia coli K-12 population only in the presence of oil-degrading microorganisms.
12. A predator-prey relationship involving heterotrophic bacteria and a protozoan was observed in Chena River water. Addition of crude oil to the water appeared to temporarily inhibit the activity of the predator.
13. Heterotrophic plate count agar was superior to nutrient agar and plate count agar for the recovery of heterotrophic bacteria from river water at 4, 10 and 20 C. Maximum recovery was obtained on spread plates after two weeks incubation at 10 C.
14. Violet red bile agar and desoxycholate agar were superior to eosin-methylene blue agar, brilliant green bile agar and tergitol 7-triphenyltetrazolium chloride agar for recovery of Escherichia coli K-12 from high numbers of non-coliform bacteria.

15. Replicate oil degradation flasks were highly variable in appearance and mutagenic activity, indicating that the microbial populations were sensitive to minor cultural differences and were relatively unstable.

The conclusions drawn from this study are presented below. The first six conclusions directly relate to the objectives of the study.

1. Naturally-occurring freshwater microorganisms are capable of mediating the production of mutagens in crude oil during microbiological degradation.
2. The conditions most favorable to mutagen production in crude oil by freshwater microorganisms are the same as those favoring microbial growth and oil degradation, that is, moderate incubation temperatures and sufficient non-hydrocarbon nutrients. Increased surface water runoff also enhances the ability of river water microorganisms to produce mutagens in crude oil.
3. Both frameshift and base-pair substitution mutagens may be produced in crude oil during microbiological degradation.
4. The aromatic fraction of crude oil contained the majority of the mutagenic activity produced by non-biological activation.

5. Freshwater microorganisms are capable of forming products in crude oil that are toxic to Escherichia coli K-12.

6. The Ames test is sufficiently sensitive and specific to study complex environmental interactions resulting in the production of mutagens in crude oil.

7. The ability of freshwater microorganisms from two geographically distinct areas to form mutagens in two compositionally different crude oils indicates that the results of this study may be applicable to other areas.

8. Microbiological activation of mutagens in crude oil in subarctic river water is apparently limited by low temperatures, insufficient non-hydrocarbon nutrients and seasonal variations in the rivers, but given the proper environmental conditions, the microorganisms in subarctic river water are capable of producing mutagens in crude oil.

9. The results of this study indicate that waters with moderate temperatures, high nutrient levels, slow turnover rates and chronic oil pollution may potentially carry an increased mutagenic burden.

10. Although it has been shown that microbiological degradation of oil may potentially increase the mutagenic burden

in water, insufficient information is available to determine if this constitutes as public health threat.

Additional research is needed to answer questions raised by this study that have not been fully investigated. New research possibilities have also been encountered. Recommendations for future studies of oil degradation by microorganisms are as follows:

1. A study employing combined activation of crude oil by microorganisms and mammalian liver microsomes is needed to determine if mutagenic activity is further enhanced by mammalian enzymes after microbial activation.
2. Solvent fraction of crude oil residues with known mutagenic activity by column chromatography is needed to determine if fractions other than the aromatics contain mutagens after microbiological degradation.
3. Factors controlling microbial succession and population variability during oil degradation need to be better defined to stabilize mixed microbial populations.
4. The microorganisms responsible for mutagen production have not been identified. The role of primary and secondary oil-degraders in mutagen production and the ability of pure cultures to produce mutagens is not known.

5. The mutagenic compounds produced in crude oil by nonbiological and microbiological processes have not been chemically characterized. This information would be of value in tracing degradation pathways leading to mutagenic activation of crude oil components.
6. The effect of crude oil on predator-prey relationships in river water could be studied more extensively.
7. The non-hydrocarbon nutrients, especially trace elements, required by microorganisms for oil degradation and mutagen production in oil should be investigated more thoroughly.
8. Oil degradation studies need to be conducted with a wide range of oils and microorganisms from a variety of habitats to determine if the microbiological production of mutagens in oil is a common occurrence in nature.
9. Additional short-term screening tests for mutagenicity and animal tests for carcinogenicity should be run on crude oil residues before and after microbial degradation to evaluate the potential health hazard of crude oil in water.

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APPENDICES

Appendix 1. Equipment list for winter water sampling in Alaska.

Ice auger motor head (full of oil-gas mixture)
Ice auger bit with extension
Ice probe (chisel point)
Shovel
Tools for auger (pliers, hammer, screwdriver)
Hip waders
Ice chest
Polyethylene dipper (UV sterilized, foil wrapped)
Polyethylene funnel (UV sterilized, foil wrapped)
Wire mesh strainer
Rope
Ice daggers
Calibrated thermometer
Rubber insulated gloves
Arm length rubber gloves
Starting ether
Notebook and pencil
Seasonal clothing
Polypropylene carboy (5 gal. with screw top, autoclaved)

Appendix 2. Composition of the three media evaluated for recovery of heterotrophic bacteria from river water.

Nutrient agar (Difco)		Plate count agar (Difco)		Heterotrophic plate count agar (Environment Canada)	
Beef extract	3.0 g	Yeast extract	2.5 g	Peptone	3.0 g
Peptone	5.0 g	Tryptone	5.0 g	Casamino acids	0.5 g
Agar	15.0 g	Dextrose	1.0 g	K_2HPO_4	0.2 g
Distilled water	1.0 l	Agar	15.0 g	$MgSO_4 \cdot 7H_2O$	0.1 g
		Distilled water	1.0 l	$FeCl_3 \cdot 6H_2O$	0.0017 g
				Agar	15.0 g
				Distilled water	1.0 l

Appendix 3. **Salts medium composition for freshwater oil degradation studies.**

NaCl	0.01 g
$MgSO_4 \cdot 7H_2O$	1.02 g
NH_4NO_3	1.0 g
KH_2PO_4 10% soln	3.0 ml
K_2HPO_4 10% soln	7.0 ml
Distilled water or river water	1.0 l
Crude oil	1.0%

Modified from medium of Walker and Colwell (349) for estuarine studies by reducing NaCl concentration 1000 times.

Water adjusted to pH 7 by proportion of mono- and dibasic potassium phosphate added. Phosphate salt solutions autoclaved separately from other salts.

Appendix 4. Characteristics of Salmonella typhimurium tester strains.

Strain characteristic	Tester strain				
	TA 100	TA 1535	TA 1537	TA 1538	TA 98
Histidine mutation	hisG46	hisG46	hisC3076	hisD3052	hisD3052
LPS mutation	galE	galE	galE	galE	galE
Excision repair mutation	uvrB	uvrB	uvrB	uvrB	uvrB
Other mutations	chl bio	chl bio	chl bio	chl bio	chl bio
R-factor pKM101	+	-	-	-	+
Histidine reversion	Base-pair missense	Base-pair missense	Frame-shift -1	Frame-shift +2	Frame-shift +2
Mutagens causing reversion	N & S Mustards MNNG	N & S Mustards MNNG	Acridines PAH	Aromatic nitroso	Aromatic nitroso

Note: strains have single deletion through galchlbiouvrB genes.

gal - galactose
chl - nitrate reductase
bio - biotin
uvrB - ultraviolet excision repair
his - histidine
MNNG - N-methyl-N-nitro-N-nitrosoguanidine
PAH - polynuclear aromatic hydrocarbons
LPS - lipopolysaccharide
N - nitrogen
S - sulfur

Appendix 5. Composition of bottom agar for mutagenicity testing.

Prepare 50X strength Vogel-Bonner E salts (344) as follows:

Distilled water	670 ml
$MgSO_4 \cdot 7H_2O$	10 g
Citric acid $\cdot H_2O$	100 g
K_2HPO_4	500 g
$NaNH_4PO_4 \cdot 4H_2O$	175 g

Add salts to distilled water with stirring in the order shown, allowing each component to dissolve completely before adding the next one. Makes approximately one liter of salts.

Prepare bottom agar (13) as follows:

Distilled water	880 ml
Vogel-Bonner E salts	20 ml
Agar	15 g
Glucose (filter sterilized)	20 g in 100 ml D.W.

Vogel-Bonner E salts are diluted 1:50 in distilled water and autoclaved with the agar. The filter sterilized glucose is added to the autoclaved medium after cooling to 50 C. Thirty ml of the melted medium is added to 100 X 15 mm petri plates and allowed to solidify.

Appendix 6. Histidine revertant response of Salmonella tester strains to standard mutagens.

Mutagen	Concn.	Tester strain				
		TA 100	TA 1535	TA 1537	TA 1538	TA 98
Methylmethane sulfonate	2 ul	+++	-	-	-	+
4-nitro-quinoline-N-oxide	10 ug	+++	+	-	+	-
N-methyl-N-nitro-N-nitroso-guanidine	2 ug	+++	+++	-	-	-
9-amino-acridine	10 ug	-	-	++	-	-
Daunomycin	10 ug	-	-	-	-	+

Mutagens were spot tested on sterile 6 mm filter paper discs.

Symbols denote approximate number of revertant colonies surrounding disc: - = less than 20 colonies, + = greater than 20 colonies (weak positive), ++ = greater than 100 colonies, +++ = greater than 500 colonies.

Appendix 7. Salmonella tester strain characterization.

Test	Condition	Tester strain				
		TA 100	TA 1535	TA 1537	TA 1538	TA 98
1. Ultraviolet light sensitivity	·Exposure time	8 sec	8 sec	6 sec	6 sec	6 sec
	Sensitivity	S	S	S	S	S
2. Ampicillin sensitivity	Zone size	0 mm	20 mm	22 mm	23 mm	0 mm
	Sensitivity	R	S	S	S	R
3. Crystal violet sensitivity	Zone size	11 mm	11 mm	12 mm	13 mm	13 mm
	Sensitivity	S	S	S	S	S
4. Biotin and histidine requirements	No addition	NG	NG	NG	NG	NG
	Biotin	2	NG	3	NG	2
	Histidine	NG	NG	NG	NG	NG
	Biotin + His	TNTC	TNTC	TNTC	TNTC	TNTC
5. Spontaneous reversions	Revertant No.	199	18	9	8	47
	Lit. value (10)	160	25	7	20	40

1. Excision repair deletion (uvrB) in all strains. One half of nutrient agar swab plate exposed to GE 30W germicidal lamp at 80 cm. Irradiated half-less than 10 colonies, unirradiated half-confluent growth.

2. R-factor (pKM101) in strains TA 100 and TA 98. 10 mcg ampicillin sensitivity disc on nutrient agar swab plate.

3. LPS deletion (rfaE) in all strains. 10 ul of 1 mg/ml crystal violet added to sterile 6 mm sensitivity disc on nutrient agar swab plate.

4. Histidine and biotin deletions in all strains. 0.1 ml of 0.1 M L-histidine or 0.1 ml of of 0.5 mM biotin spread on Vogel-Bonner E bottom agar streak plates. NG - no growth, TNTC - too numerous to count, number - spontaneous histidine revertants.

5. Spontaneous histidine revertants on mutagenicity test plates without mutagens. Average of three plates.

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